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Host jump in BPVs: is species-specificity still appropriate for papillomaviruses?

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1 General characteristics

1.1 History

In many cultures, warts have been associated with magic and a multitude of folk cures, due to their sudden appearance and disappearance (Bunney et al., 1992). Already in the writings of the Byzantine physician Aetius of Amida (sixth century AD), it was possible to find the description of papillomatous lesions: ‘‘The term thymus arises from the similarity to the tips of the plant of the same name, which grows in the mountains. It appears in the anus and the pudenda and between the legs’’. Aulus Cornelius Celsus, who lived during the reign of Tiberius Caesar, in discussing wart-like lesions in his book “De Medecina” described three morphologically distinct types of cutaneous warts, but the most common term 'verruca' was firstly used by the German Daniel Sennert (1572–1637) in his book entitled “Hypomnemata physicae” (1636) describeing that “they appear on the surface of the skin like the eminences of little hills”.

In the past, theories about the causation of warts abounded: in 1849, a medical section of the *Lancet* stated that “Dr Durr frequently observed warts on index and middle finger of females addicted to solitary habit”, alluding to masturbation. Folklore in fact provides a rich source of theories of the cause of warts, like repeated wetting of the hands, washing the hands in water in which eggs have been boiled, the killing of a toad, the foam of the sea-shore and contact with animals, particularly cows and chickens.

Obviously, popular belief on treatment of warts abounded, including animal, plant or mineral remedies consisting of local applications of fish heads, pig's blood, pig's fat, lizard's blood, menstrual blood, dog's dung, dove's dung, tobacco juice and fasting spittle (Bett, 1951).

It was only in 1823 that Sir Astley Cooper introduced the concept of a transmissible agent as responsible for wart causation, while in 1891 Joseph Payne recorded their contagious nature, subsequently confirmed with inoculation experiments (Variot, 1894; Jadassohn, 1896). The probable viral origin of warts was suggested by Ciuffo who produced warts on his hands by inoculating himself with a wart extract filtered in order to exclude bacteria and fungi, in 1907; subsequently in 1919 Wile and Kingery transmitted warts in human beings, and Kingery (1921) a couple of years later was also able to produce them in the second generation with filtrates.

HPV virus particles in warts were firstly demonstrated on EM in 1949 by Strauss and colleagues but only in 1962 Melnick classified the wart virus in the Papova virus group.

So far, observations of warts had been reported in different animal species (Penbertby 1898; Cook and Olson 1951; Moulton, 1954; Shope and Hurst 1933; Lucke et al 1950), and in the 70s it was determined that the causative agents were several diverse viruses.

Contagiousness of warts in animals was recognized at the beginning of the XX century in cattle and water buffalo (Cadeac, 1901; Royer, 1902; Schindelka, 1903; Da Costa, 1906) where it was noticed that the subject could develop a fibropapilloma after inoculation of scarified skin with the bovine papilloma agent. In such lesions, the demonstration of the presence of a filtrable agent was performed only in 1929 (Creech), while in 1933 the so called CRPV (Cottontail Rabbit Papillomavirus) was recognized as the first DNA oncovirus and was also the first animal PV to be identified by Shope. In cattle it was later observed that the virus was also responsible for the development of fibropapilloma of the genital mucosa (Mcentee, 1952) and a polyp-like tumor in the urinary bladder (Olson et al., 1959), as well as a sarcoma-like condition in the skin of the horse (Olson et al., 1951). While in 1959 it was also shown that regressing tumors of the bladder could be induced by wart extracts, demonstrating that BPVs cause bladder neoplasia (Olson et al., 1959).

In humans, Human Papilloma Virus (HPV) has been identified as a major public health problem to such an extent that the 2008 Nobel Prize in Physiology or Medicine went to Harald zur Hausen, who discovered the role that HPV plays in the pathogenesis of cervical cancer, prompting much research on the nature and mechanism of papillomavirus-induced oncogenesis, which in turn has advanced understanding of papillomavirus infections in animals. In fact, the virus infectious cycle and the neoplastic progression from papilloma to carcinoma are broadly similar in humans and animals (Table 1), and animal PVs and their hosts represent excellent model systems for HPVs, infection and neoplastic progression (Borzacchiello et al., 2009; Campo, 2002) as well as vaccination study in the natural host.

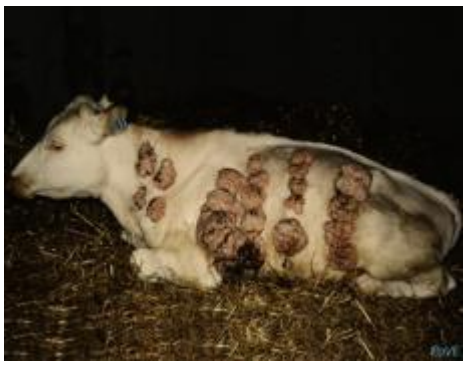

Figure 1- This cow was inoculated with BPV1 virus by Carl Olson at the University of Wisconsin. The warts were harvested by Carl Baker and Paul Lambert and the material obtained (virus, tissue sections and RNA) was used for many of the key studies of BPV1 biology.

Observations on BPVs made 50 years ago	Subsequently determined relevance in HPVs
BPVs may cause cancer	HPVs now estimated to cause 5% of human cancer
BPV infection shows difference manifestation depending on host factors	The immune response of the host strongly influences the manifestation of HPV infection
BPVs can infet cells other than mucocutaneous epithelial cells	A diverse range of cancer are currently being investigated for a possivle manifestation of HPV infection
Determining the significance of BPV in a tumor can be difficult	While HPVs have been detected in many different human cancers, their ubiquitous nature makes it hard to assign significance
BPVs can infect multiple species	Although rare, cross-species infections by HPVs has been reported

Table 1- Observations on Bovine Papillomaviruses (BPVs) made prior to or in the paper by Cheville and Olson, published in veterinary pathology in 1964, and the subsequently determined relevance to Human Papillomavirus (HPV) biology. Modified from Munday et al. 2014.

1.2 Taxonomy

The actual classification of the papillomaviruses into the family *Papillomaviridae* was proposed in the 7th ICTV report (2000) and entered into force in 2003 (de Villiers, 2013). In fact, formerly, papillomaviruses and polyomaviruses were grouped into the family *Papovaviridae* based on their structural similarities, but later it was demonstrated that they have different genome sizes, completely different organizations, and no major nucleotide or amino acid sequence similarities except for the presence of some local homologies. PVs' taxonomy in the past has been performed in many ways: comparing panels of restriction digests, via cross-wise southern blot hybridizations, under non stringent conditions, and liquid hybridizations, furthermore, PVs do not elicit robust antibody responses in the course of natural infections, which impeded a classification based on "serotype" designations.

Determination of PV genotypes is nowadays based on their degree of homology, where the frequency distribution of pairwise identity percentages of L1 genes demonstrates three main taxonomic levels, namely genera, species and types (de Villiers et al., 2004).

Different genera, designated by Greek letters, share less than 60% nucleotide sequence identity in the L1 ORF and less than 23% to 43% when their full genomic sequences are compared. The classification of PVs into genera unites several phylogenetically related species, previously called ''supergroups'' or ''major branches'' (Myers et al., 1994; Chan et al., 1995), that differ with respect to their biological properties. Different species within the same genus exhibit 60-70% similarity in their L1 ORFs. The term ''species'' is biologically useful, as these are natural *taxa* based on a close phylogenetic relationship of certain types and because such species typically lump PV types which have common biological and pathological properties, which is a requirement of ICTV guidelines. A new viral type must have a completely cloned genome and the DNA sequence of the L1 region must differ by >10% of the closest known type. Nomenclature is based on the scientific name of the host species followed by a progressive number, based on the publication date of the virus and/or

the submission of the sequence to a public database and deposit of the cloned viral genome. Only the human and bovine papillomaviruses are designated with the historical abbreviations "HPV" (with H standing for human or Homo, but avoiding the species designation "sapiens") and "BPV" (with B standing for bovine or Bos, but avoiding the species designation "taurus") respectively (Bernard et al., 2010). Reference type species are identified either because they are the most comprehensively investigated type, or because they represent best the species, or because there is only one type in that taxon. Papillomavirus genomes with a nucleotide sequence identity between 2% and 10% from the L1 ORF in the reference genome have been named subtypes, while less than 2% define a variant. Recently the ICTV Papillomavirus Study Group proposed a classification and nomenclature criteria for papillomavirus variants (Chen et al., 2011) considering the use of full genome sequences. This is derived from the fact that isolates of the same type are closely related and the full extent of the sequence heterogeneity is best summed across the whole genome and recently evolved variant genomes have changes that are not always evenly distributed throughout the genome (Burk et al. 2011). Currently, 49 genera, from *Treisdeltapapillomavirus* to *Dyoomegapapillomavirus* comprise over 318 fully characterized PV types (http://pave.niaid.nih.gov, last access 26 dec 2015). Around 180 of them are human isolates, reflecting the fact that human PVs have been extensively studied compared with animal PVs (Figure 2).

The four different genera *Delta Epsilon Xi* and *Dyoxi* include the fifteen BPVs (BPV 1-15) that have been characterized so far in cattle, even though it has been estimated that type number may exceed 20 (Lunardi et al., 2013b).

The *Delta* papillomaviruses comprise the ruminant papillomaviruses, in particular BPV types 1, 2, 13 and 14, whose peculiarity is to cause benign fibropapillomas starting from the transformation of subepithelial fibroblasts followed by epithelial acanthosis and then papillomatosis. The ability of BPV1 and BPV2 to non-productively transform non-epithelial cells is not species-specific, although the increased host range is limited to fibroblasts as

further shown by the induction of experimental fibroblastic tumors in hamsters, and focal transformation of cultured rodent cells (Cheville, 1966). I*n vivo*, BPV1, 2 and 13 are the only papillomaviruses able to cause natural infections outside the primary host causing the development of sarcoids in horses (Nasir and Campo, 2008; MacLachlan and Dubovi, 2011; Lunardi et al., 2013a, b).

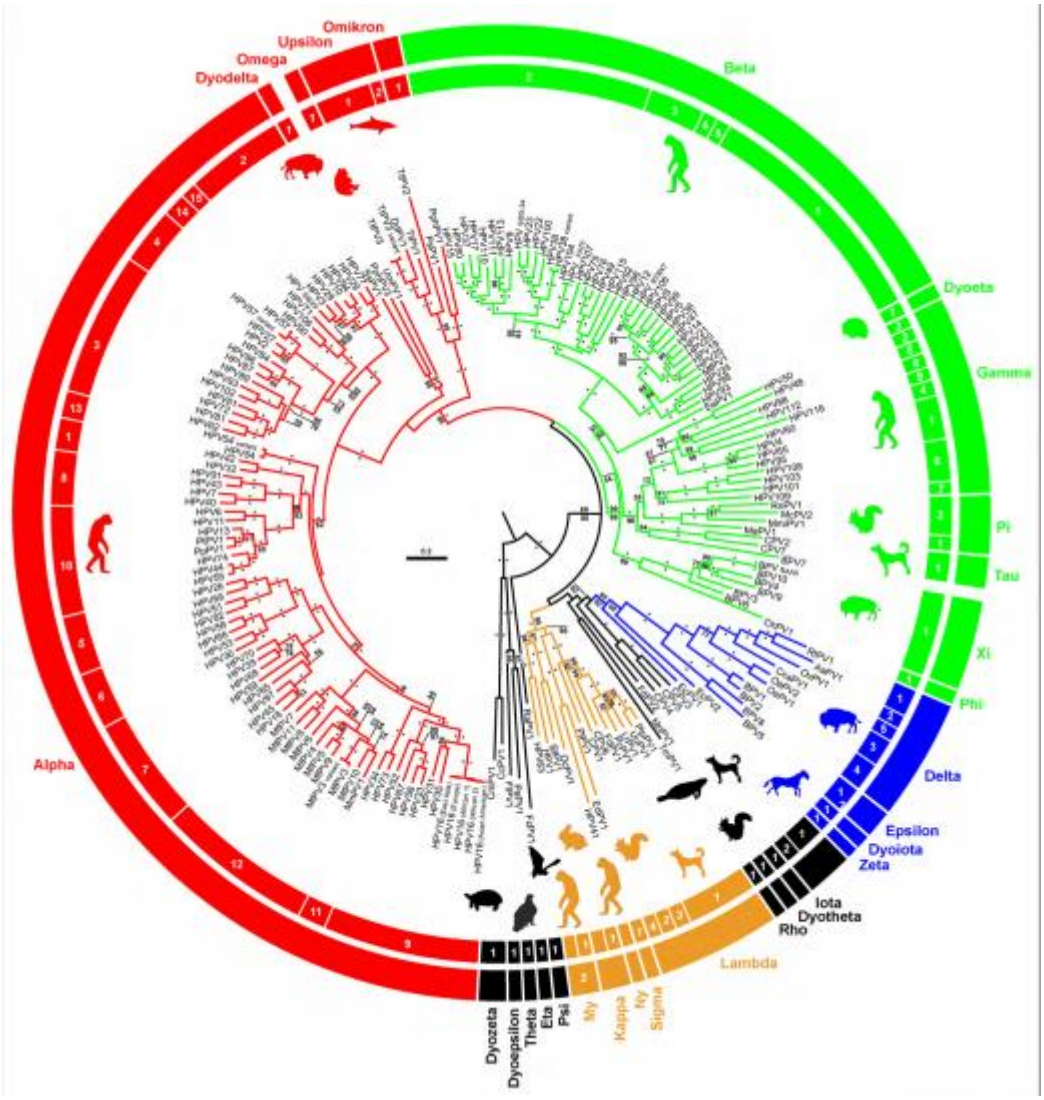

Figure 2; the best-known maximum likelihood phylogenetic tree for PVs. This tree was constructed using the concatenated E1–E2–L1 genes, aligned at the amino acid level. Color codes highlight the four PV supertaxa:. PVs that cannot be assigned yet with confidence to a supertaxon are labeled in black. Silhouettes represent the hosts infected by the corresponding viruses. Adapted from Bravo et al., 2010

Following its first identification, BPV14 was formerly classified as feline sarcoid-associated papillomavirus (FeSarPV), but phylogenetic analyses revealed that its sequence was most

similar to the *Deltapapillomavirus* species BPVs 1, 2, and 13, while it was distantly related to any carnivoran PV (Munday et al., 2010). Since FeSarPV sequence has never been detected in any non-sarcoid sample from cats but has been amplified from the skin of cattle, it was hypothesized that feline sarcoids develop due to cross-species infection of cats by a bovine *Delta*-PV as cattle are commonly reared in all countries where feline sarcoids have been previously reported (Schulman et al., 2001).

Epsilonpapillomaviruses comprise BPV5 and 8 whose genomes seem to share similarities with both *Xi*-PVs and *Delta*-PVs (Bloch and Breen, 1997) and at the same time they appear to have a dual pathology causing both fibropapillomas and epithelial papillomas (Bloch et al., 1994). *Xipapillomaviruses* include BPV3, 4, 6, 9, 10, 11, 12 and 15 that induce epithelial papillomas (de Villiers et al., 2004; Hatama et al., 2008, 2011; Zhu et al., 2012) with transformation only of the epithelial component.

The last genus comprises only BPV7, that has been isolated for the first time in Japan (Ogawa et al., 2007), and has been classified as *Dyoxipapillomavirus* (Rector and Van Ranst, 2013).

Anyhow, this classification should be considered a guideline and cannot be intended definitive since Batista and colleagues (2013) reported a more frequent occurrence of co-infections and a possible association of pure epitheliotropic types with cutaneous fibropapillomas.

1.3 Virus structure

Like other papillomaviruses, BPV virions are non enveloped, spherical 55 nm in diameter with a T=7d icosahedral capsid composed of 360 L1 molecules that can self-assemble into 72 pentamers (capsomers). The neighboring capsomeres form the capsid that contains a single molecule of circular covalently closed, supercoiled dsDNA associated with histones with a size up to ~9 Kbp (Figure 3). Both full (Figure 4) and empty virus particles are seen by electron microscopy.

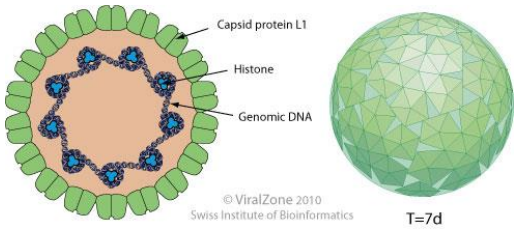

Figure 3: Papillomaviral structure. Source: ViralZone: www.expasy.org/viralzone.
SIB Swiss Institute of Bioinformatics

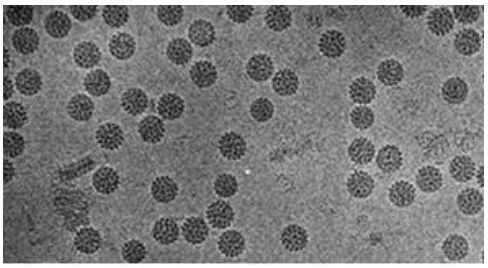

Figure 4: Virus Like Particles

Papillomaviruses are resistant to diverse environmental insults: infectivity survives lipid solvents and detergents, low pH, and high temperatures (50°C, 1 hr). Neither lipids nor carbohydrates are present, while the virus genomes encode 8-10 proteins with sizes ranging from 7-73 kDa (Figure 5). The encoding ORFs (Open Reading Frame) located on a single DNA strand can be divided into early (E) and late (L) depending on the time of viral DNA replication occurrence. Papillomaviruses show a remarkable conservation in the overall genomic organization (Figure 4): the elements shared fom the different genera of the family *Papillomaviridae* are the LCR (Long Control Region) and the E1, E2, L1, L2 ORFs (Vallvè et al., 2005; Rector and Van Ranst, 2013).

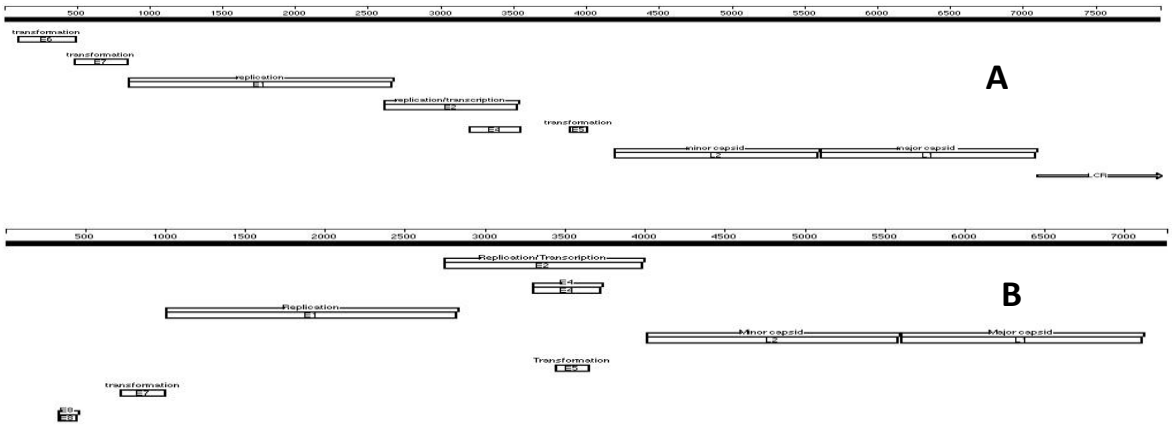

Figure 5- Molecular organization of BPV1 (A), considered the typical prototype architecture of the papillomavirues, and BPV4 (B) genomes. In BPV4, the E6 is replaced by an open reading frame originally termed E8 which later was renamed E5 because of its structural and functional similarities to BPV-1 E5 (Jackson et al., 1991; Faccini et al., 1996).

Although gene number is limited by the small size of the papillomavirus genome, gene expression involves the use of multiple promoters and complex patterns of splicing, increasing the number of encoded proteins.

L1 and L2 make up the capsid and are expressed only in the fully differentiated keratinocytes at the surface of the papilloma (Borzacchiello and Roperto, 2008), the remaining are non-structural proteins (E1-E8). The transformation process is modulated by the three oncogenes, E5, E6 and E7, while E1 and E2 modulate transcription and regulation processes (Munger and Howley, 2002). The untranslated LCR region contains the origin of replication and the binding sites of multiple transcription factors (Zheng and Baker, 2006) and appears to mutate at a faster rate than other parts of the PV genome (Rector et al., 2007).

1.4 Evolution

The fact that PV's phylogeny resembles to some extent the phylogeny of their hosts (Rector, et al. 2007; Bernard, 2005) suggest that the main driving factor of PV diversification over millions of years (Halpern 2000; Bernard et al., 2006) is the ‘‘host-linked evolution’’ (Chan et al. 1992, 1995).

An ancestral, probably cutaneotropic PV, might have diverged into a small number of different PVs linked to the differentiation of mammalian skin (Garcia-Vallve, 2005). However, if co-divergence alone was the driving force for the observed PV diversity, then (i) all PVs infecting the same host should be monophyletic and (ii) the topologies of the phylogenetic trees of hosts and of PVs should be entirely congruent (Bravo et al., 2010). As a matter of fact, barely half of the known host–parasite associations appear to derive from co-phylogenetic events (Gottschling et al., 2011).

Evolutionary incongruence might arise from singular events in the past such as the establishment of new ecological niches, and/or asymmetric genome convergences driven by intense selection (Narechania et al. 2005; Varsani et al. 2006), but also within-host virus

duplication, viral sorting, or viral adaptation after a host switch, may contribute considerably to PV diversification (Shah et al., 2010; Gottschling et al, 2011).

In particular, evolution upon host switch could ultimately lead to the colonization of a new host (Antia et al., 2003). This seems to have been the case with BPV1 and BPV2 in horses, where infection can be a result of human domestication of cattle and horses or a phenotypic acquisition driven by vector-mediated interspecies transmission (Finlay et al., 2009; Gottschling et al., 2011). Thus, concominant ecological changes in the different hosts may have increased their susceptibility to BPV cross-infection and/or augmented the frequency of physical contact between them to grant BPV improved access to a potential new host (Gottschling et al., 2011). Anyhow, the genetic changes in the viral sequences suggest an adaptation process to the environment provided by the new host (Nasir and Campo, 2008). More examples for heterologous PV infections include the presence of feline sarcoma PV on bovine skin (Munday and Knight, 2010), the presence of HPV9 in lesions of a cat (Munday et al. 2007), the possible transient infections of zookeepers with animal PVs (Antonsson and Hansson, 2002) and interspecies infection between macaques (Chen et al., 2009).

Nevertheless, the rare occurrence of recombination events is also documented. Besides experimental infection in the rabbit model (Hu et al., 2009), in nature it has been reported for cetacean papillomaviruses, with recombination breakpoints closely corresponding to the early and late genomic regions (Gottschling et al., 2011; Rector et al., 2008; Robles-Sikisaka et al., 2012), but also the clinically important *Alpha*PVs (i.e. HPV16, 18) have undergone recombination event(s) between the early and the late regions of the genome (Bravo and Alonso, 2004; Narechania et al., 2005; Carvajal-Rodriguez 2008). Surprisingly, recombination has also been reported between the two families *Polyomaviridae* and *Papillomaviridae* in the chimeric viruses retrieved from western barred bandicoot (*Perameles bougainville*) (Woolford et al. 2007).

2 Interaction of Bovine Papillomaviruses with the cell

2.1 Replication cycle

The knowledge regarding papillomavirus replication is originally based on the ability of BPV1 to infect and transform the mouse fibroblasts cell line (C127) allowing the virus to establish and maintain genomes as extrachromosomal DNA with a constant copy number (Lancaster, 1981; Law et al., 1981). Infection does not require the virus capsid, as skin abrasion and exposure to the naked viral genome are able to recapitulate the complete natural history of the infection in different animal models (Cladel et al., 2008). Papillomaviruses infect the mitotically active basal cells, whose replication maintains the infection; subsequent cell differentiation triggers the production of the PV early proteins, preventing the suprabasal cell from leaving the cell cycle (which would result in nuclear degeneration) and instead causing the cell to re-enter the S-phase and produce numerous viral copies. Indeed genome is delivered into the nucleus and is episomally maintained at a low copy number. The productive infectious cycle needs differentiation and keratinization of the infected cells: early and late genes are expressed in a temporal and highly regulated manner leading to the formation of lesions that are usually benign and tend to regress, and therefore considered hyperplastic rather than neoplastic (Munday, 2014).

In particular, in the basal and lower levels of the epithelium, the early genes encoding for pro-mitotic proteins and viral replication factors are expressed, while terminal cell differentiation is required for activation of late gene expression and production of viral particles at the very top of the epithelium. Virions are shed into the environment from the uppermost layers of the epithelium where keratinocytes undergo terminal differentiation steps that ultimately lead to desquamation. The life cycle is therefore intra-epithelial, produces no cell lysis, or cell death, and replication is not associated with inflammation (Stanley, 2012).

PVs are taken up by the cell through endocytosis following primary attachment to the host cell, subsequently the uncoating program starts at the plasma membrane by binding to heparan sulfate proteoglycan (HSPG) receptors and initiating the first structural changes within the capsid.

The mechanisms of papillomavirus endocytosis have not been fully resolved for all the different PV types; it mostly seems like L1 and L1/L2 particles initially traffic similarly, passing through early endosomes to late endosomes (Day et al., 2003; Kämper et al., 2006). In the latter, the acidification seems also to facilitate the dissociation of the L1 protein from the L2/DNA complex in the endosomal compartment (Bienkowska-Haba et al., 2012). Trafficking occurs on average 2–3 h post internalization indicating prolonged residence in the endosomal system (Schelhaas et al., 2012). After this step, L2 and the genome are transferred to the trans-Golgi network prior to nuclear delivery (Day et al., 2013).

After entry into the nucleoplasm, expression of E1 and E2 results in the production of 10 to 200 episomal PV copies (Maglennon et al., 2011) and maintaining the keratinocyte in a proliferative state. Gene expression is regulated at the level of transcription (Bernard, 2002;) and RNA processing (Schwartz, 2008; Zheng and Baker, 2006). Viral replication is performed by high-fidelity cellular polymerases, in parallel to the replication of the cellular genome (Doorbar et al., 2012) under the mediation of E1, E2, E4 and E5 viral proteins. E1 and E2 are involved in papillomavirus replication and in intragenomic regulation, E5, E6 and E7 induce cellular DNA replication, E4 may represent a late function and binds to specific cytoskeleton structures. An important difference in the *Delta*-BPVs is the predominance of the E5 protein's activity compared to E6 and E7 in the cell cycle's disruption during a productive infection (Bohl et al., 2001; Silva et al., 2013). It has also emerged that intra-type sequence variation that occurs within papillomavirus types, can influence the cellular location and function of the oncoproteins and consequently affect the pathogenesis and transforming ability of the virus (Giannoudis and Herrington, 2001).

Transcription of the early and late coding regions occurs from the same strand in one direction only; precursor mRNAs undergo post-transcriptional processing that includes capping and polyadenylation of the 5´- and 3´-termini, respectively, and the papillomavirus proteins are produced from a myriad of alternatively spliced and polyadenylated mRNAs (Schwartz et al., 2013). Advantages of the extensive use of alternative splicing and poly-adenylation include the ability to express many genes from a compact genome, as well as the ability to individually regulate the expression of each gene during the viral life cycle.

Furthermore, papillomaviruses cause the depletion of intraepithelial Langerhans cells, which are crucial for T-cell priming (Leong et al., 2009; Matthews et al., 2003), downregulation of the surface major histocompatibility complex (MHC) class I, the major presenter of antigenic peptides to T cells (Ashrafi et al., 2006a, b), and interference with type I interferon (IFN) signaling (Antonsson et al., 2006; Frazer et al., 1999). As a consequence, infections often go unnoticed by the host also explaining the long persistence of the papillomas, even in immunocompetent individuals.

2.2 Latency

The latent stage of the life cycle of a virus is typified by its presence in the absence of clinical signs of disease. It is unclear whether HPV detection, after antibody response, reflects virologic clearance or establishment of viral latency (Gravitt, 2011) but it is considered likely that papillomaviruses cause latent infections (Broker et al., 2001; Nicholls et al., 2001; Stubenrauch and Laimins, 1999). In fact, PV genome can be detected in healthy skin both in human and animals by PCR (Antonsson e Hansson, 2002; Ogawa et al., 2004) demonstrating latent or subclinical infections (Antonsson et al., 2000; Astori et al., 1998; Boxman et al., 1997) where the production of new virus particles is restricted, and the viral genome is maintained in a silent state with little or no gene expression. The undifferentiated proliferating keratinocytes, presumably stem cells, are the initial target for productive PV infections and

establishment of latency (Kadaja et al., 2009); however, viral genomes can also reside in lymphocytes, both in cattle and in humans (Campo et al., 1994a; Chen et al., 2009b). Latent infection of lymphocytes has also been established experimentally in cattle (Stocco dos Santos et al., 1998).

Reactivation generally allows the formation of a productive infection, with or without the reemergence of a visible lesion (Rajcani, 2007) and seems to be related to skin trauma with subsequent production of inflammatory citokines and cellular proliferation. In humans, the high post-treatment recurrence rates of genital papillomas caused by low-risk HPV types 6 and 11 (Lacey, 2005) and the identification of DNA and RNA transcripts in clinically healthy tissues of recurrent respiratory papillomatosis affected patients (Abramson et al., 2004; Maran et al., 1995; Pignatari et al., 1992; Smith et al., 1993) seems to support this hypothesis.

It is difficulte to directly demonstrate establishment of latency and induction of reactivation from the latent state in studies involing human subjects. This has driven investigators to make use of animal models of infection, but the limited availability of well-characterized rodent models, has meant that most previous work on papillomavirus latency has focused on larger domesticated animals, and in particular on rabbits, dogs and cattle.

Cottontail rabbit papillomavirus (CRPV) can induce an asymptomatic infection, characterized by the persistence of viral DNA in the absence of visible signs of disease (Amella et al., 1994; Zhang et al., 1999) with the production of E1 transcripts. The detection of viral DNA and RNA at regressed sites appears to be largely confined to the basal epithelial cells, and is not generally associated with completion of the virus life cycle and the production of new virions but the virus may be capable of reactivation under certain conditions (Maglannon et al., 2011).

Similarly, it has been suggested that canine oral papillomavirus (CPV 1) may cause a latent infection with DNA detected following the regression of papillomas in the absence of clinical signs of disease associated to low genome copy number (Stanley et al., 2001). As a mucosal

virus, CPV1 has been proposed as an animal model of HPV 6 and 11- associated RRP (Recurrent respiratory papillomatosis) in humans (Jahan-Parwar et al., 2003), CPV1 has also been used in vaccine development (Johnston et al., 2005; Moore et al., 2002; Stanley et al., 2001) and immune response to infection investigations (Nicholls et al., 2001; Jain et al., 2006).

An 'endogenous infection' of the Mastomys Natalensis by the MnPV (Mastomys Natalensis Papillomavirus) is demonstrated by the presence of DNA at low copy number in the skin in the absence of clinical signs of disease. In fact, following repeated chronic mechanical irritation of the skin with fine glasspaper over a period of eight weeks, a 58-fold increase in viral DNA copy number was detected compared to non-irritated skin (Siegsmund et al., 1991). The infection of the multimammate rat appears more interesting from a laboratoristic pont of view. Laboratory mice indeed provide numerous advantages as a biomedical tool over the current PV models in that they are relatively safe, easy to handle, and numerous immunological and biological reagents are readily available for detailed molecular studies (Ingle et al., 2011).

The virus was demonstrated to be transmissible to immunocompetent mice; this characteristic made it also possible to do simple transmission studies and to investigate the pathogenesis of this infection and possibly determine the key genetic/protein components of resistance, susceptibility, and even progression to squamous cell carcinoma

BPV entry may also result in an asymptomatic, latent infection (McBride, 2008), as it has been identified within BPV4-associated urinary bladder cancers (Campo, 1997; Campo et al., 1992; Borzacchiello et al., 2003b). This viral type has also been frequently detected in normal bovine bladder suggesting its presence as a latent infection that can undergo reactivation when cattle graze on pastures rich in bracken fern (Smith, 1997) but may even be due to some other factors such as infection with bovine viral diarrhoea virus (Tsirimonaki et al., 2003). When latent BPV is activated, full malignant transformation depends on other mutagens such as

quercetin and ptaquiloside that act synergistically with the virus in the carcinogenic process, triggering BPV gene expression and leading to the development of cancer (Borzacchiello, 2007).

In horses where BPV 1 and 2 DNA can be detected in the normal skin, the occurrence of sarcoids at sites of skin wounding and/or following physical trauma suggests a possible reactivation from latency (Carr et al., 2001; Bogaert et al., 2008).

In different species, reactivation has been correlated to exposure to ultraviolet light (Zhang et al., 1999), but also suppression of the adaptive immune system can lead directly to an elevation of the viral DNA copy number at sites of previous infection, which is consistent with reactivation from latency (Maglannon et al., 2014).

3 Interaction of Bovine Papillomaviruses with cattle

3.1 Epidemiology

Bovine papillomaviruses are related to serious livestock diseases causing significant economic losses in both beef and dairy cattle enabling the progression to cancer with or without the action of cofactors. Their worldwide distribution is documented by a number of infections in regions with a great density of cattle like Italy, United Kingdom, Germany, Japan, India, United States of America and Brazil (Campo and Jarrett, 1986; Ogawa et al., 2004; Singh et al., 2009; Schmitt et al., 2010; Carvalho et al., 2012).

Even if it is not stringent, studies on the molecular epidemiology of BPVs correlated the identification of the different virus types with particular tissue tropism and specific lesions.

As a result, BPV-1 has been related to teat (Figure 6), penile and cutaneous fibropapillomas (Figure 7), BPV- 2 to skin warts and gastrointestinal (GI) fibropapillomas, BPV-3 to skin papillomas, BPV-4 to epithelial papillomas of GI tract and skin, BPV-5 to udder fibropapillomas, BPV- 6 to teat papillomas, BPV-7 and BPV-8 to cutaneous warts, BPV-9 and BPV-10 to epithelial squamous papilloma lesions on cattle teats, BPV-11 to teat fibropapillomas and the last identified, BPV-13, has been associated with cutaneous papilloma of the ear (Ogawa et al., 2007; Borzacchiello and Roperto, 2008; Hatama et al., 2011; Lunardi et al., 2012; Zhu et al., 2012; Batista et al., 2013).

Figure 6: multiple papillomatous lesions close to

Figure 7: cutaneous papilloma on the lower lid of a cow the papillary ostium

Warts are detected in around one-third of cattle at slaughter (Lindholm et al., 1984), cattle of any age can be affected but younger animals are more prone to lesions' development showing a spontaneous regression within one year (Jelínek and Tachezy 2005) due to the animal's immune response. Indeed, immunosuppression predisposes to PV infection progression and can be caused by factors such as infection with bovine viral diarrhea virus (Tsirimonaki et al., 2003) and stress factors like gestation and lactation, to cause extensive and persistent papilloma formation (Munday et al., 2014). Rates of disease of upper alimentary tract papillomas are dependent on immunosuppressants present in bracken fern: 20% of cattle from geographical areas with this plant will have papillomas compared with just 4.4% of cattle from areas without this plant (Jarrett et al., 1978; Jensen et al., 1981).

3.2 Diseases, transmission and therapy

BPV is consequently relevant as an agent of disease in farm animals and therefore of considerable veterinary and agricultural importance (Campo, 2002). Papillomas tend to regress, but can occasionally persist and provide the focus for malignant transformation to squamous cell carcinoma once the virus synergizes with environmental carcinogenic co-factors.

Viral transmission can occur via direct or indirect contact between infected animals or with contaminated areas, such as milking machines, water dispensers, feeders, ropes or fences, or transmitted by insects (Love et al., 2012). BPV infections may start from a micro-injury, which exposes the peptidoglycan of heparin sulfate present in the plasma membrane (McBride et al., 2012). Some authors suggest that the blood can act as a reservoir for BPV (Freitas et al., 2003; Brandt et al., 2008) and lymphocytes can harbor the virus in the bloodstream (Stocco dos Santos et al., 1998; Freitas et al., 2003; Diniz et al., 2009) which can spread through non-epithelial tissues and fluids. Parallely to HPV, the transplacental

transmission of PVs appears to take place in cattle (Freitas et al., 2003; Rombaldi et al., 2008; Roperto et al., 2012).

Benign neoplasm

Warts are commonly detected in cattle and have been reported to be caused by BPV types belonging to the *Delt*a, *Xi*, *Dyoxi*, and *Epsilon* genera (Rector and Van Ranst 2013).

Muco-cutaneous papillomas develop on the haired skin, tongue, teats, penis, vulva and upper alimentary papillomas in the oral cavity, esophagus, and rumen.

Papillomas can be experimentally induced by inoculating scarified skin with either BPV-1 or BPV-2 (Jarrett et al., 1990; Olson et al., 1992) but develop only at the site of inoculation, suggesting that any BPV DNA circulating within lymphocytes does not produce additional cutaneous lesions.

BPV infection is considered necessary for the development of GI papillomas that can develope in every site from the mouth to the rumen, this can subsequently induce difficulty in feeding and breathing that may be fatal (Campo, 1997; Tsirimonaki et al., 2003; Dong et al., 2013; Gordon, 1997).

Papillomas and fibropapillomas on the genital area, teats (Figure 8), prepuce and penis result in difficulties in milking, suckling and mating, (Nasir and Campo, 2008; Jarrett,1985b). Usually papillomas persist for 1-6 months before spontaneous immune-mediated response (Knowles et al., 1996), but some animals are unable to reject the infection and succumb to widespread cutaneous or mucosal involvement and tumours may undergo neoplastic transformation.

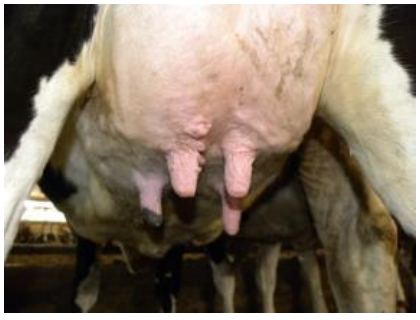

Figure 8: papillomas on teats and udder in a dairy cow

Malignant neoplasm

Campo (1997) demonstrated the association of BPV-4 with GI papilloma and squamous cell carcinoma, this discovery was subsequently supported by different findings (Campo et al., 1980; Borzacchiello et al., 2003b). Papillomas persist and may be transformed into malignant carcinomas, including squamous cell carcinomas (Hamada et al., 1989; Campo et al., 1994b; Borzacchiello et al., 2003b; Masuda et al., 2011).

Urinary bladder cancer comprises two main types that often occurr together in the same subject, carcinoma of the urothelium and hemangioendotheliomas of the subjacent capillaries. In general, BPV-2 has been detected in urinary bladder tumours (Borzacchiello et al., 2003a), and it is known to be involved in the aetiology of chronic enzootic haematuria (CEH) both in buffaloes and in cattle due to prolonged ingestion of bracken fern (Campo et al., 1992). In subjects suffering from the disease, chromosomal instability in the form of chromosomal aberrations was detected, confirming the synergistical action of the different component (Lioi et al., 2004). Interestingly, Roperto and colleagues (2012) also identified BPV-1/-2 DNA in the placenta of pregnant cows suffering from CEH.

4 Interaction of Bovine Papillomaviruses with horse

4.1 Epidemiology of sarcoid

Equine sarcoids are the most common dermo-epithelial skin tumors in horses and other equids like donkeys, mules and zebras worldwide (Jackson, 1936; Ragland, 1970; Lazary et al., 1985; Nel et al., 2006; Bogaert et al., 2008; Nasir and Campo, 2008) with a variable prevalence ranging from 1 to 8% (Knottenbelt, 2005). The tumour is locally invasive, refractory to treatment but is often highly invasive at multiple sites and can lead to the animal being sacrified, with morbidity rates reported as high as 14 per 1000 horses (Campo, 2002; Mohammed et al., 1992). The skin of the head, ventral abdomen groin and legs of horses have been reported as the most commonly affected regions of the body (Jackson, 1936; Ragland et al., 1970, Strafuss et al., 1973, Lane 1977, Fretz and Barber 1980). A higher tendency of sarcoid development has been registered in regions with a hot climate (Shokry et al., 2015). In fact, there is strong circumstantial evidence that flies may be able to transmit BPV-1 between equids (Finlay et al., 2009) and it is possible that the different biting flies in different geographical areas have some role in the regional variations in numbers and types of sarcoid.

Regarding lesions'distribution, in the UK it is relatively rare to find horses with single or few lesions, as multiple lesions (10 to several thousand) are much more common here than elsewhere. On the contrary, in Australia, it is less common to find horses with large numbers of sarcoid respect to continental Europe and North America, where an average of two or eight sarcoid per horse is described (Knottenbelt et al., 1995). A predisposition is observed in geldings (Mohammed et al., 1992) and also in males in donkeys (Reid et al., 1994; Reid and Gettinby, 1996). The wound is assumed as a possible entrance for BPV (Reid et al., 1994), on the other hand the castration wound itself is only seldom affected. However most authors do not assume that there is a gender predisposition for sarcoid development (Ragland, 1970; Miller and Campbell, 1982; Pulley and Stannard, 1990; Torrontegui and Reid, 1994).

Nevertheless, certain breeds and families are more vulnerable than others (Ragland et al., 1966; Brostrom et al., 1988; Lazary, 1988; Gerber, 1989; Marti et al., 1993): in NorthAmerica, Quarter Horses are twice as likely to develop sarcoids compared to Thoroughbreds (Angelos et al., 1988). Also Appaloosas and Arabian horses are more frequently affected than Thoroughbreds (Angelos et al., 1988; Mohammed et al., 1992), this could be linked to the higher risk of these breed to get in contact with BPV working with cattle (Mohammed et al., 1992). Moreover, Jackson (1936) demonstrated a predilection for those areas of the animal's body which are frequently subjected to trauma, and specifically Quarter Horses and Appalossas often work on rough surfaces resulting in frequent injury to the limbs (Brostrom, 1995). Standardbreds on the other hand are rather resistant (Meredith et al., 1986; Mohammed et al., 1992; Brostrom, 1995).

Equine sarcoids predominantly develop in young adults: the mean age of sarcoid development is 3.5 to 4 years (Brostrom, 1995; Studer et al., 2007), but a gradual increase in incidence is also observed up to the age of 15 years, followed by a decreasing incidence (Mohammed et al. 1992). This distribution might be correlated to the acquisition of a certain level of immunity in adults which results in spontaneous regression of existing tumors and prevention of new tumor development. Another hypothesis is that genetically susceptible animals develop tumors early in life (Torrontegui and Reid, 1994).

Genetic background is supposed to be involved in sarcoid epidemiology, for example, strong familial tendencies to sarcoid have been described (James, 1968; Marti et al., 1993). Genetic predisposition has been identified with over 80% of affected horses being MHC class I alleles (Lazary et al., 1985; Meredith et al., 1986). The genes of the MHC code for proteins involved in the immune response and for protein components of the complement system (Piscopo, 1999). The suggested autosomal recessive gene responsible for imparting a susceptibility to the condition probably influences the severity and recurrence of the disorder in individuals.

4.2 Disease, transmission and therapy

Papillomaviruses are generally considered to be highly specific for their hosts; however, within the *Delta*-PVs, BPV types 1, 2 and 13 are well recognized to infect multiple species causing sarcoid tumours in horses, donkeys and mules (Olson and Cook, 1951). Infections are also described in other distantly related mammals like buffaloes, yaks, tapirs, giraffes, bisons and zebras (Lohr et al., 2005; Literak et al., 2006; Kidney and Berrocal, 2008; Nasir and Campo, 2008; Silvestre et al., 2009; Pangty et al., 2010; van Dyk et al., 2011; Bam et al., 2012; Kumar et al., 2013). The economic loss is important both for the racing industry and even more for the farmers in developing countries, for whom often the mule or donkey is the only livelihood.

Although the pathology has been recognized for centuries (Erk, 1976), the term sarcoid was firstly used by Jackson in 1936 who described the lesion as "a unique locally invasive, benign neoplastic like tumour of the skin with a variable epidermal component which has a high propensity for recurrence". The name itself, meaning flesh-like was used to emphasise the clinical and pathological differences from papilloma, fibroma, and fibrosarcoma and to suggest the malignant sarcomatous appearance of the lesions. However, various terms have been used to describe sarcoids, including warts papillomas, fibrosarcomas, schwannomas and recurring granulation tissue.

Macroscopically, sarcoids show a variable appearance and have been classified in to six distinct clinical forms (Pascoe and Knottenbelt, 1999). Verrucous sarcoids have a typical wartlike appearance with a rough, thickened, scabby surface above the fibroblastic part of the tumor (Knottenbelt et al., 1995); nodular sarcoids (Figure 9) are subcutaneous, easily moveable nodules, often but not always spherical, covered by intact, apparently normal skin (Foy et al., 2002); fibroblastic sarcoids are large fibrous masses with an ulcerated surface; occult sarcoids (Figure 9) are flat circular to oval areas characterized by alopecia and a roughened or scaly appearance (Knottenbelt et al., 1995); malevolent (Figure 10) sarcoids

infiltrate in lymphatic vessels resulting in multiple nodular or fibroblastic masses along these vessels; mixed sarcoids are a conglomerates of two or more sarcoid types in various combinations. Categorization of sarcoid lesions is important because the various clinical forms may require different treatment approaches, and it also helps in professional discussions to have a common approach to the description of the various types (Knottenbelt, 2005).

BPV DNA has been detected in up to 100% of examined sarcoid tumours (Otten et al. 1993; Carr et al. 2001; Martens et al. 2001a, b; Bogaert et al. 2007), with a majority of BPV1 DNA compared to BPV2 DNA, which may reflect geographical distribution (Angelos et al., 1991; Otten et al., 1993; Martens et al., 2001b; Bogaert et al,. 2007; Wobeser et al., 2010). Interestingly, sequence analysis of BPV DNA isolates extracted from sarcoids demonstrated the presence of distinct equine sarcoid-specific variants of BPV (Otten et al., 1993; Reid et al., 1994; Chambers et al., 2003; Nasir et al., 2007). The sequence changes in the E5 protein reported by Reid and colleagues (1994) and Chambers and colleagues (2003) suggest the possibility that these changes are contributing factors to the pathogenesis of the disease.

The predominant cell type is a malignant/transformed fibroblast but the histological components of the equine sarcoid can include, in variable proportions, fibroblasts, epidermis, and endothelial cells.

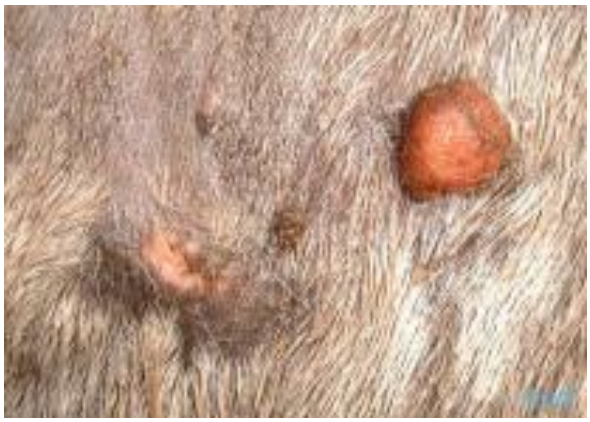

Figure 9: occult (hairless area, left) and nodular (large roundbump at right) forms of equine sarcoid. Source: PaVE. Author: Malcom Morely.

Figure 10: Malignant sarcoid on the abdomen of a horse

It is important to recognize that the “less severe” forms can rapidly progress to the more aggressive types with infiltration of surrounding skin, particularly if they are traumatized (Marti et al., 1993; Broström, 1995; Knottenbelt et al., 1995, 2003; Ragland, 1970).

Generally in different sarcoid types, the epidermis varies from thick, rough and hyperkeratotic to thinned or ulcerated while in less typical sarcoids the epidermal component can be normal, atrophic or even absent (Lepage et al., 1998; Marti et al., 1993; Martens et al., 2000).

Diagnosis of equine sarcoids can be assessed in three different ways: biopsy followed by histopathological analysis, which represents the definitive differentiation method but is not without hazard; the others are detection of BPV DNA, and clinical examination (Martens et al., 2000; Bogaert et al., 2008; Carr, 2009; Sala et al., 2010). The latter can be difficult to assess as other conditions can macroscopically be hard to differentiate from sarcoids.

Transmission between horses and eventually from cattle to horses is still controversial. Horse-to-horse transmission was suggested by the findings of specific BPV-1 variants in sarcoid lesions which are not present in BPV-1 infections in cattle (Nasir et al., 2007); direct contact and rubbing have been implicated, but also vertical transmission via blood and semen of horses may take place (Freitas et al. 2003; Yaguiu et al. 2008). Moreover, it is believed that flies play a role in the transference of BPV between cattle and horses (Nasir and Campo, 2008; Finlay et al., 2009).

Treatment is often challenging, due to the variable clinical presentation of lesions and the frequent local recurrences, therefore various topical applications (Stadler et al., 2011), immunotherapy (Klein 1990, 1986; Theon 1998), radiation therapy (Henson and Dobson 2004; Theon et al., 1995; Byam-Cook et al., 2006), chemotherapy (Theon et al., 1999; Spoormakers et al., 2003; Knottenbelt et al., 2000), cryosurgery (Martens et al., 2001c; Carr 2009; Scott and Miller 2011), ‘traditional’ sharp surgery (Knottenbelt et al., 2000; Reschke, 2012) and laser surgery (Diehl et al., 1987; Vingerhoets et al., 1988; Carstanjen et al., 1997), have all been reported in the veterinary literature and an even wider variety of nonprescription

treatments are used by the horse-owning public (Knottenbelt and Walker, 1994; Sonvico et al., 2009). Different methods have been used separately, or have been combined, with variable efficacy. If lesions are small and few in numbers or where they do not interfere with normal function it may be better not to intervene therapeutically. This also applies to cases considered too severe to be treated by any conventional means, but where other factors such as pregnancy, may be a reason for prolonging life. Therefore, vaccinations and immunotherapies have been also explored as they may stimulate the immune system to limit the viral spread.

5 References

- Abramson, A.L., Nouri, M., Mullooly, V., Fisch, G., Steinberg, B.M., 2004. Latent human papillomavirus infection is comparable in the larynx and trachea. J. Med. Virol. 72 (3), 473–477.
- Amella, C.A., Lofgren, L.A., Ronn, A.M., Nouri, M., Shikowitz, M.J., Steinberg, B.M., 1994. Latent infection induced with cottontail rabbit papillomavirus, A model for human papillomavirus latency. Am. J. Pathol. 144 (6), 1167–1171.
- Angelos, J., Oppenheim, Y., Rebhun, W., Mohammed, H., Antczak, DF. 1988. Evaluation of breed as a risk factor for sarcoid and uveitis in horses. Anim Genet.;19(4):417-25
- Angelos, J.A., Marti, E., Lazary, S. and Carmichael, L.E. 1991. Characterization of BPV-like DNA in equine sarcoids. Arch. Virol. 119, 95-109.
- Antia, R., Regoes, RR., Koella, JC., Bergstrom, CT. 2003. The role of evolution in the emergence of infectious diseases. Nature. 11;426(6967):658-61.
- Antonsson, A., Forslund, O., Ekberg, H., Sterner, G., Hansson, BG. 2000. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. J Virol. Dec;74(24):11636-41.
- Antonsson, A., E. Payne, K. Hengst, and N. A. McMillan. 2006. The human papillomavirus type 16 E7 protein binds human interferon regulatory factor-9 via a novel PEST domain required for transformation. J. Interferon Cytokine Res. 26:455–461;
- Antonsson, A., Hansson, B.G. 2002. Healthy skin of many animal species harbors papillomaviruses which are closely related to their human counterparts. J. Virol. 76, 12537–12542
- Ashrafi, G. H., Brown, D. R., Fife, K. H., Campo, M. S. 2006a. Downregulation of MHC class I is a property common to papillomavirus E5 proteins Virus Res. 120:208–211.
- Ashrafi, G. H., Mohammad, H., Marchetti, B., Campo, M. S. 2006b. E5 protein of human papillomavirus 16 downregulates HLA class I and interacts with the heavy chain via its first hydrophobic domain. Int. J. Cancer 119: 2105–2112.
- Astori, G., Lavergne, D., Benton, C., Höckmayr, B., Egawa, K., Garbe, C., de Villiers, EM. 1998. Human papillomaviruses are commonly found in normal skin of immunocompetent hosts. J Invest Dermatol. May;110(5):752-5.
- Bam, J., Kumar, P., Leishangthem, G. D., Saikia, A., Somvanshi, R. 2012. Spontaneous cutaneous papillomatosis in yaks and detection and quantification of bovine papillomavirus -1 and -2. Transbound. Emerg. Dis. 60, 475–480.
- Batista, M.V.A., Silva, M.A.R., Pontes, N.E., Reis, M.C., Corteggio, A., Castro, R., Borzacchiello, G., Balbino, V.Q., Freitas, A.C. 2013. Molecular epidemiology of bovine papillomatosis and the identification of a putative new virus type in brazilian cattle. Vet. J. 197 (2), 368-373.
- Bernard, H.U., Burk, R.D., Chen, Z., van Doorslaer, K., zur Hausen, H., de Villiers, E.M. 2010. Classification of papillomaviruses based on 189 PV types and proposal of taxonomic amendments. Virology; 401 (1) 70-79.
- Bernard, HU., Calleja-Macias, IE., Dunn, ST. 2006. Genome variation of human papillomavirus types: phylogenetic and medical implications. Int J Cancer. Mar 1;118(5):1071-6.
- Bernard, H.U. 2005. The clinical importance of the nomenclature, evolution and taxonomy of human papillomaviruses. J. Clin. Virol. 32S, S1–6
- Bernard, H.U., 2002. Gene expression of genital human papillomaviruses and considerations on potential antiviral approaches. Antiviral Ther. 7, 219–237.
- Bett, WR. 1951 'Wart, I bid thee begone.' Practitioner. 166:77-80
- Bienkowska-Haba, M., Williams, C., Kim, SM., Garcea, RL., Sapp, M. 2012. Cyclophilins Facilitate Dissociation of the HPV16 Capsid Protein L1 from the L2/DNA Complex Following Virus Entry. J Virol 86(18):9875–9887
- Bloch, N., Breen, M., Irvin, Z.V., Spradbrow, P.B. 1996. Bovine papillomavirus type 4 DNA isolated from a skin lesion in a steer. Veterinary Record; 138 (17), 414-416.
- Bloch, N., Sutton, R.H., Spradbrow, P.B. 1994. Bovine cutaneous papillomas associated with bovine papillomavirus type 5. Archives of Virology; 138 (3-4), 373-377.
- Bogaert, L., Van Poucke, M., De Baere, C., Dewulf, J., Peelman, L., Ducatelle, R., Gasthuys, F., Martens, A. 2007. Bovine papillomavirus load and mRNA expression, cell proliferation and p53 expression in four clinical types of equine sarcoid. J Gen Virol. 88(Pt 8):2155-61..
- Bogaert, L., Martens, A., Van Poucke, M., Ducatelle, R., De Cock, H., Dewulf, J., De Baere, C., Peelman, L. & Gasthuys, F. 2008. High prevalence of bovine papillomaviral DNA in the normal skin of equine sarcoid-affected and healthy horses. Vet Microbiol 129, 58–68.

- Bohl, J., Hull, B., Vande Pol, SB. 2001. Cooperative transformation and coexpression of bovine papillomavirus type 1 E5 and E7 proteins. J Virol.75:513–521.
- Borzacchiello, G., Iovane, G., Marcante, ML., Poggiali, F., Roperto, F., Roperto, S., Venuti, A. 2003a. Presence of bovine papillomavirus type 2 DNA and expression of the viral oncoprotein E5 in naturally occurring urinary bladder tumours in cows. J Gen Virol. .84(Pt 11):2921-6.
- Borzacchiello G., Ambrosio V., Roperto S., Poggiali F., Tsirimonakis E., Venuti A., Campo M.S., Roperto F. 2003b. Bovine papillomavirus type 4 in oesophageal papillomas of cattle from the south of Italy. J. comp. Pathol128:203–206
- Borzacchiello, G., Roperto, F., Nasir, L., Campo, M. S. 2009. Human papillomavirus research: do we still need animal models? Int. J. Cancer 125:739–740.
- Borzacchiello, G., Iovane, G., Marcante, M. L., Poggiali, F., Roperto, F., Roperto, S., Venuti, A. 2003a: Presence of bovine papillomavirus type 2 DNA and expression of the viral oncoprotein E5 in naturally occurring urinary bladder tumours in cows. J. Gen. Virol. 84, 2921–2926.
- Borzacchiello, G. 2007. Bovine papillomavirus infections in animals. Communicating Current Research and Educational Topics and Trends in Applied Microbiology A. Méndez-Vilas (Ed.)
- Borzacchiello, G., Roperto, F. 2008: Bovine papillomaviruses, papillomas and cancer in cattle. Vet. Res. 39, 45.
- Boxman, IL., Berkhout, RJ., Mulder, LH., Wolkers, MC., Bouwes Bavinck, JN., Vermeer, BJ., ter Schegget, J. 1997. Detection of human papillomavirus DNA in plucked hairs from renal transplant recipients and healthy volunteers. J Invest Dermatol. 108(5):712-5.
- Brandt, S., Haralambus, R., Schoster, A., Kirnbauer, R., Stanek, C. 2008. Peripheral blood mononuclear cells represent a reservoir of bovine papillomavirus DNA in sarcoid-affected equines. J Gen Virol. Jun;89(Pt 6):1390-5. doi: 10.1099/vir.0.83568-0.
- Bravo, IG., Alonso, A. 2004. Mucosal human papillomaviruses encode four different E5 proteins whose chemistry and phylogeny correlate with malignant or benign growth. J Virol;78:13613–26
- Bravo, IG., de Sanjosé, S., Gottschling, M. 2010. The clinical importance of understanding the evolution of papillomaviruses. Trends Microbiol. 18(10):432-8. doi: 10.1016/j.tim.2010.07.008.
- Broker, TR., Jin, G., Croom-Rivers, A., Bragg, SM., Richardson, M., Chow, LT., Vermund, SH., Alvarez, RD., Pappas, PG., Squires, KE., Hoesley, CJ. 2001. Viral latency--the papillomavirus model. Dev Biol (Basel).;106:443-51;
- Brostrom, H. 1995. A clinical and epidemiologic study in relation to equine leukocyte antigens (ELA). Acta Vet Scand 36, 223-236
- Brostrom, H., Fahlbrink, E., Dubath. M.L., Lazary, S. 1988. Association between equine leukocyte antigens (ELA) and equine sarcoid tumors in the population of swedish halfbreds and some of their families. Veterinary Immunol Immunop 19, 215-223.
- Broström, H: Equine Sarcoids: A Clinical, Epidemiological and Immunological study. PhD Thesis, University of Uppsala, Stockholm, Sweden, 1995
- Bunney, M.H., Benton, C., Cubie, H.A., 1992. Viral Warts: Biology and Treatment. 2nd Ed Oxford University Press.
- Burk, R. D., Chen, Z., Harari, A., Smith, B. C., Kocjan, B. J., Maver, P. J., Poljak, M. 2011. Classification and nomenclature system for Human Alphapapillomavirus variants: general features, nucleotide landmarks and assignment of HPV6 and HPV11 isolates to variant lineages Acta Dermatovenerol Alp Panonica Adriat.. 20(3): 113–123.
- Byam-Cook, KL., Henson, FM., Slater, JD. 2006. Treatment of periocular and non-ocular sarcoids in 18 horses by interstitial brachytherapy with iridium-192. Vet Rec;159(11):337–41.
- Caedac, 1901. Sur la transmission experimentale des papillomes des especes. Bull. Soc. Sci. Vet. de Lyon. 1: 280-286.
- Campo, M.S. 2006a. Bovine papillomavirus: old system, new lessons? In Campo M.S. (ed.) Papillomavirus research: from natural history to vaccines and beyond. Caister Academic Press, UK, 373-387.
- Campo, M.S. 1994a. Latent papillomavirus infection in cattle. Research in Veterinary Science; 56 (2), 151-157.
- Campo, M.S., Moar M.H., Jarrett W.F.H., Laird H.M. 1980. A new papillomavirus associated with alimentary cancer in cattle. Nature. 286:180–182.
- Campo, MS., Jarrett, WF., Barron, R., O'Neil, BW., Smith, KT. 1992. Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. Cancer Res. 15;52(24):6898-904.
- Campo, MS., O'Neil, BW., Barron, RJ., Jarrett, WF. 1994b. Experimental reproduction of the papilloma-carcinoma complex of the alimentary canal in cattle. Carcinogenesis.;15(8):1597-601.
- Campo, M. S. 2002: Animal models of papillomavirus pathogenesis. Virus Res. 89, 249–261.
- Campo, M. S., 1997. Bovine papillomavirus and cancer. Vet. J. 154, 175–188.
- Campo, M. S., Jarrett, W. F. 1986. Papillomavirus infection in cattle: viral and chemical cofactors in naturally occurring and experimentally induced tumours. Ciba Found. Symp. 120, 117–135.

- Carr, EA. New developments in diagnosis and treatment of equine sarcoid. In: Robinson NE, Sprayberry KA, editors. Current therapy in equine medicine. 6th edition. St Louis (MO): Saunders Elsevier; 2009. p. 698.
- Carr, E.A., Theon, A.P., Madewell, B.R., Hitchcock, M.E., Schlegel, R. and Schiller, J.T. 2001. Expression of a transforming gene (E5) of bovine papillomavirus in sarcoids obtained from horses. Am. J. Vet. Res. 62, 1212-1217.
- Carstanjen, B., Jordan, P., Lepage, OM. 1997. Carbon dioxide laser as a surgical instrument for sarcoid therapy--a retrospective study on 60 cases. Can Vet J. Dec;38(12):773-6.
- Carvajal-Rodriguez, A. 2008. Detecting recombination and diversifying selection in human alpha-papillomavirus. Infect Genet Evol;8:689–92
- Carvajal-Rodriguez, A. 2008. Detecting recombination and diversifying selection in human alpha-papillomavirus. Infect Genet Evol;8:689–92.].
- Carvalho, C. C., Batista, M. V., Silva, M. A., Balbino, V. Q., Freitas, A. C. 2012. Detection of bovine papillomavirus types, co-infection and a putative new BPV11 subtype in cattle. Transbound. Emerg. Dis. 59, 441–447
- Celsus, Aulus Cornelius. De Medicina - with an English translation by WG Spencer. Book V 28;14 1962;Vol.2: 160-3 4
- Cerqueira C, Liu Y, Kühling L, Chai W, Hafezi W, van Kuppevelt TH, Kühn JE, Feizi T, Schelhaas M. Heparin increases the infectivity of Human Papillomavirus type 16 independent of cell surface proteoglycans and induces L1 epitope exposure. Cell Microbiol. 2013 Nov;15(11):1818-36. doi: 10.1111/cmi.12150.
- Chambers, G., Ellsmore, V.A., O'Brien, P.M., Reid, S.W.J., Love, S., Campo, M.S., Nasir, L. 2003. Sequence variants of bovine papillomavirus E5 detected in equine sarcoids. Virus Res; 96 (1-2), 141-145
- Chan, S.Y., Delius, H., Halpern, A.L., Bernard, H.U. 1995. Analysis of genomic sequences of 95 papillomavirus types: uniting typing, phylogeny, and taxonomy. J. Virol. 69, 3074–3083.
- Chan, S.-Y., Bernard, H.-U., Ong, C.-K., Chan, S.-P., Hofmann, B., Delius, H. 1992. Phylogenetic analysis of 48 papillomavirus types and 28 subtypes and variants: a showcase for the molecular evolution of DNA viruses. J. Virol. 66:5714–5725
- Chen, AC., Keleher, A., Kedda, MA., Spurdle, AB., McMillan, NA., Antonsson, A. 2009b. Human papillomavirus DNA detected in peripheral blood samples from healthy Australian male blood donors. J Med Virol. Oct;81(10):1792-6. doi: 10.1002/jmv.21592.
- Chen, Z., Schiffman, M., Herrero, R., Desalle, R., Anastos, K., Segondy, M., Sahasrabuddhe, VV., Gravitt, PE., Hsing, AW., Burk,.RD. 2011. Evolution and taxonomic classification of human papillomavirus 16 (HPV16)-related variant genomes: HPV31, HPV33, HPV35, HPV52, HPV58 and HPV67. PLoS One. 6(5):e20183. doi: 10.1371/journal.pone.0020183.
- Chen, Z., van Doorslaer, K., DeSalle, R., Wood, CE., Kaplan, JR., Wagner, JD., Burk, RD. 2009. Genomic diversity and interspecies host infection of alpha12 Macaca fascicularis papillomaviruses (MfPVs). Virology 25;393(2):304-10. doi: 10.1016/j.virol.2009.07.012.
- Cheville, NF., Olson, C. 1964. Epithelial and fibroblastic proliferation in bovine cutaneous papillomatosis. Vet Pathol.;1:248–257
- Cheville, NF. 1966. Cancer research 28 Part 1, 2334-2339
- Ciuffo, G. 1907. Innesto positivo con filtrato di verruca volgare. G Ital Mal Vener 42:12-17
- Cladel, NM., Hu, J., Balogh, K., Mejia, A., Christensen, ND. 2008. Wounding prior to challenge substantially improves infectivity of cottontail rabbit papillomavirus and allows for standardization of infection. J Virol Methods. Mar;148(1-2):34-9.
- Cook, R. H., Olson, C. 1951. Experimental transmission of cutaneous papilloma of the horse. Am. J. Pathol. 97: 1087-1097. 14.
- Cooper Sir Astley. On warts. Lancet 1823+4;il:303-5
- Creech, G. T. 1929. Experimental studies of the etiology of common warts in cattle. J. Agric. Res. 39: 723-73
- Da Costa, J. 1906. Papillomatous tumours in a buffalo. Vet. Rec. 19: 570.
- Day, PM., Thompson, CD., Schowalter, RM., Lowy, DR., Schiller, JT. 2013. Identification of a role for the trans-Golgi network in human papillomavirus 16 pseudovirus infection. J Virol.;87(7):3862-70. doi: 10.1128/JVI.03222-12.
- Day, PM., Lowy, DR., Schiller, JT. 2003. Papillomaviruses infect cells via a clathrin-dependent pathway. Virology. 1;307(1):1-11.
- de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U., zur Hausen, H. 2004. Classification of papillomaviruses. Virology; 324 (1), 17-27.
- de Villiers, EM. 2013. Cross-roads in the classification of papillomaviruses. Virology. Oct;445(1-2):2-10. doi: 10.1016/j.virol.2013.04.023.

- Diehl, M., Vingerhoets, M., Stornetta, D. 1987. Spezifische Methoden zur Entfernung des Equine Sarkoides. Der Praktische Tierarzt, Collegium Veterinarium 18, 14-16.
- Diniz, N., Melo, TC., Santos, JF., Mori, E., Brandão, PE., Richtzenhain, LJ., Freitas, AC., Beçak W, Carvalho RF, Stocco RC. 2009. Simultaneous presence of bovine papillomavirus in blood and in short-term lymphocyte cultures from dairy cattle in Pernambuco, Brazil. Genet Mol Res. 15;8(4):1474-80. doi: 10.4238/vol8-4gmr668.
- Dong, J., W. Zhu, Y. Goto, and T. Haga, 2013. Initial detection of a circular genome deletion in a naturally bovine papillomavirus-infected sample. J. Vet. Med. Sci. 75, 179–182
- Doorbar, J., Quint, W., Banks, L., Bravo, IG., Stoler, M., Broker, TR., Stanley, MA. 2012. The biology and life-cycle of human papillomaviruses. Vaccine. 20;30 Suppl 5:F55-70. doi: 10.1016/j.vaccine.2012.06.083.
- Erk, N. 1976. A study of Kitab al-Hail wal-Baitar, written in the second half of the ninth century by Muhammed Ibn ahi Hizam. Hist Vet 1:101-104,
- Faccini, A.M., Cairney, M., Ashrafi, G.H., Finbow, M.E., Campo, M.S., Pitts, J.D. 1996. The bovine papillomavirus type 4 E8 protein binds to ductin and causes loss of gap junctional intercellular communication in primary fibroblasts. J Vir; 70 (12), 9041-9045
- Finlay, M., Yuan, Z., Burden, F., Trawford, A., Morgan, I. M., Campo, M. S., Nasir, L. 2009: The detection of Bovine Papillomavirus type 1 DNA in flies. Virus Res. 144, 315–317.
- Foy, J.M., Rashmir-Raven, A.M., Brashier, M.K. 2002. Common equine skin tumors. Compendium on Continuing Education for the Practicing Veterinarian 24, 242.
- Frazer, I. H., Thomas, R., Zhou, J., Leggatt, G. R., Dunn, L., McMillan, N., Tindle, R. W., Filgueira, L., Manders, P., Barnard, P., Sharkey, M. 1999. Potential strategies utilised by papillomavirus to evade host immunity. Immunol. Rev. 168:131–142
- Freitas, A.C., Carvalho, C., Brunner, O., Birgel-Junior, E.H., Dellalibera, A.M.M.P., Benesi, F.J., Gregory, L., Beçak, W., Stocco dos Santos, R.C. 2003. Viral DNA sequences in peripheral blood and vertical transmission of the virus: a discussion about BPV-1. Brazilian Journal of Microbiology; 34 (1), 76-78.
- Freitas, 2013. Molecular epidemiology of bovine papillomatosis and the identification of a putative new virus type in Brazilian cattle. Vet. J. 197, 368–373.
- Fretz, P.B., Barber, S.M. 1980. Prospective analysis of cryosurgery as the sole treatment for equine sarcoids.Vet Clin N AmSmall 10, 847-859.
- García-Vallvé, S., Alonso, A., Bravo, IG. 2005. Papillomaviruses: different genes have different histories. Trends Microbiol. 13(11):514-2.
- Gerber, H. 1989. The Genetic-Basis of Some Equine Diseases. Equine Vet J 21, 244-248.
- Giannoudis, A., Herrington, CS. 2001. Human papillomavirus variants and squamous neoplasia of the cervix. J Pathol. Mar;193(3):295-302.
- Goodrich, L., Gerber, H., Marti, E., Antczak, DF. 1998. Equine sarcoids. Vet Clin North Am Equine Pract. Dec;14(3):607-23, vii.
- Gordon, PJ. 1997. Surgical removal of a fibropapilloma from the reticulum causing apparent vagal indigestion. Vet Rec. 140:69–70
- Gottschling, M., Göker, M., Stamatakis, A., Bininda-Emonds, OR., Nindl, I., Bravo, IG. 2011. Quantifying the phylodynamic forces driving papillomavirus evolution. Mol Biol Evol. 28(7):2101-13. doi: 10.1093/molbev/msr030.
- Gravitt, PE. 2011. The known unknowns of HPV natural history. J Clin Invest. Dec;121(12):4593-9. doi: 10.1172/JCI57149.
- Halpern, AL. 2000. Comparison of papillomavirus and immunodeficiency virus evolutionary patterns in the context of a papillomavirus vaccine. J Clin Virol.;19(1-2):43-56.
- Hamada, M., Oyamada, T., Yoshikawa, H., Yoshikawa, T. 1989. Morphological studies of esophageal papilloma naturally occurring in cattle. Nihon Juigaku Zasshi. ;51(2):345-51.
- Hatama, S., Ishihara, R., Ueda, Y., Kanno, T., Uchida, I. 2011. Detection of a novel bovine papillomavirus type 11 (BPV-11) using xipapillomavirus consensus polymerase chain reaction primers. Archives of Virology; 156 (7), 1281-1285
- Hatama, S., Nobumoto, K., Kanno, T. 2008. Genomic and phylogenetic analysis of two novel bovine papillomaviruses, BPV-9 and BPV-10. J. of Gen. Vir. 89 (1), 158-163.
- Hatama, S., R. Ishihara, Y. Ueda, T. Kanno, and I. Uchida, 2011. Detection of a novel bovine papillomavirus type 11 (BPV-11) using xipapillomavirus consensus polymerase chain reaction primers. Arch. Virol. 156, 1281–1285
- Henson, F.M.D., Dobson, J.M. 2004. Use of radiation therapy in the treatment of equine neoplasia. Equine Vet Educ 16, 315-318.

- Howley, P. M., and D. R. Lowy. 2007. Papillomaviruses, p. 2299-2354. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 5th ed., vol. 2. Lippincott Williams and Wilkins, Philadelphia, PA.
- Hu, J., Cladel, NM., Budgeon, L., Balogh, KK., Christensen, ND. 2009. Papillomavirus DNA complementation in vivo. Virus Res. 144(1-2):117-22. doi: 10.1016/j.virusres.2009.04.006.
- Ingle, A., Ghim, S., Joh, J., Chepkoech, I., Bennett Jenson. A., Sundberg, JP. 2011. Novel laboratory mouse papillomavirus (MusPV) infection. Vet Pathol.;48(2):500-5. doi: 10.1177/0300985810377186. Jackson, C. 1936. The incidence and aethology of tumors of domesticated animals in South Africa. Onderstepoort J. Vet. &i. Animal Indust. 6: 1-460.
- Jackson, M.E., Pennie, W.D., McCaffery, R.E., Smith, K.T., Grindlay, G.J., Campo, M.S. 1991. The B subgroup bovine papillomaviruses lack an identifiable E6 open reading frame. Mol Carcinogen; 4 (5), 382-387.
- Jadassohn, J. 1896. Sind die Verrucae vulgares ubertragbar? Verhandl Deutsch Dermat Gesellsch; 5:497
- Jahan-Parwar, B., Chhetri, DK., Hart, S., Bhuta, S., Berke, GS. 2003. Development of a canine model for recurrent respiratory papillomatosis. Ann Otol Rhinol Laryngol. 112(12):1011-3.
- Jain, S., Moore, RA., Anderson, DM., Gough, GW., Stanley, MA. 2006. Cellmediated immune responses to COPV early proteins. Virology, 356:23-34.
- James, V.S. 1968. A familial tendency to equine sarcoids. Southwest Vet 21; 235-236
- Jarrett, W. F. 1985a. The natural history of bovine papillomavirus infections. In: Klein G. (ed.) Advances in Viral Oncology, 5th edition. Raven Press, USA, 83-101.
- Jarrett, WF., Murphy, J., O'Neil, BW., Laird, HM. 1978. Virus-induced papillomas of the alimentary tract of cattle. Int J Cancer. Sep 15;22(3):323-8.
- Jarrett, WF., O'Neil, BW., Gaukroger, JM., Laird, HM., Smith, KT., Campo, MS. 1990. Studies on vaccination against papillomaviruses: a comparison of purified virus, tumour extract and transformed cells in prophylactic vaccination. Vet Rec. 5;126(18):449-52.
- Jarrett, WF., O'Neil, BW., Gaukroger, JM., Laird, HM., Smith, KT., Campo, MS. 1990. Studies on vaccination against papillomaviruses: a comparison of purified virus, tumour extract and transformed cells in prophylactic vaccination. Vet Rec. 5;126(18):449-52.
- Jarrett, W. F. 1985b: Bovine papillomaviruses. Clin. Dermatol. 3, 8–19.
- Jelinek, F., Tachezy, R. 2005. Cutaneous papillomatosis in cattle. J Comp Pathol.;132:70–81
- Jensen, R., Lauerman, LH., England, JJ., Braddy, PM., Horton, DP., Flack, DE., Cox, MF., Einertson, N., Miller, GK., Rehfeld, CE. 1981. Laryngeal diphtheria and papillomatosis in feedlot cattle. Vet Pathol.;18:143–150
- Johansson, C., Somberg, M., Li, X., Backström Winquist, E., Fay, J., Ryan, F., Pim, D., Banks, L., Schwartz, .S . 2012. HPV-16 E2 contributes to induction of HPV-16 late gene expression by inhibiting early polyadenylation. The EMBO Journal; 31(14): 3212–3227;
- Johnston, K.B., Monteiro, J.M., Schultz, L.D., Chen, L., Wang, F., Ausensi, V.A., Dell, E.C., Santos, E.B., Moore, R.A., Palker, T.J., Stanley, M.A., Jansen K.U. 2005. Protection of beagle dogs from mucosal challenge with canine oral papillomavirus by immunization with recombinant adenoviruses expressing codon-optimized early genes Virology. 336:208–218
- Kadaja, M., Silla, T., Ustav, E., Ustav, M. 2009. Papillomavirus DNA replication – from initiation to genomic instability. Virology. 20;384(2):360-8. doi: 10.1016/j.virol.2008.11.032.
- Kämper, N., Day, PM., Nowak, T., Selinka, HC., Florin, L., Bolscher, J., Hilbig, L., Schiller, JT., Sapp, M. 2006. A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes. J Virol. 80(2):759-68.
- Kidney, B. A., Berrocal, A. 2008. Sarcoids in two captive tapirs (Tapirus bairdii): clinical, pathological and molecular study. Vet. Dermatol. 19, 380–384
- Kingery, L. B. 1921. The etiology of common warts. Their production in the second generation. JouF. Amer. Med. Assoc. 76: 440-442, illus.
- Klein, W.R. 1990. Immunotherapy in squamous-cell carcinomas in the bovine eye and equine sarcoids. Tijdschrift Voor Diergeneeskunde 115, 1149-1155.;
- Klein, W.R., Bras, G.E., Misdorp, W., Steerenberg, P.A., Dejong, W.H. 1986 .Tiesjema R.H., Kersjes A.W., Ruitenberg E.J.. Equine sarcoid - BCG immunotherapy compared to cryosurgery in a prospective randomized clinical-trial. Cancer Immunol Immun 21, 133-140.;
- Knottenbelt, DC., Edwards, S., Daniel, E. 1995. Diagnosis and treatment of the equine sarcoid. In Practice 17, 123-129
- Knottenbelt, DC., Kelly, DF. 2000. The diagnosis and treatment of periorbital sarcoid in the horse: 445 cases from 1974 to 1999. Vet Ophthalmol.;3(2-3):169-191.

- Knottenbelt, DC. 2003. Manual of Equine Wound Management. London, WB Saunders
- Knottenbelt, DC. Walker, J. A. 1994. Treatment of the equine sarcoid. Equine Vet Educ 6, 72–75.
- Knottenbelt, DC. 2005. A Clinical Classification of the Equine Sarcoid. Clinical Techniques in Equine Practice 4; 278-295
- Knowles, G., O'Neil, B. W., Campo, M. S. 1996. Phenotypical characterization of lymphocytes infiltrating regressing papillomas. J. Virol. 70, 8451–8458
- Kumar, P., Nagarajan, N., Saikumar, G., Arya, R. S., Somvanshi, R. 2013. Detection of bovine papilloma viruses in wart-like lesions of upper gastrointestinal tract of cattle and buffaloes. Transbound. Emerg. Dis. 63, 56–67
- Lacey, C.J. 2005. Therapy for genital human papillomavirus-related disease. J. Clin. Virol. 32 (Suppl 1), S82–S90.
- Lancaster, W. D. 1981. Apparent lack of integration of bovine papillomavirus DNA in virus- induced equine and bovine tumor cells and virus-transformed mouse cells. Virology 108, 251-5
- Lane, J. G. 1977. The treatment of equine sarcoids by cryosurgery. Equine Veterinary Journal 9, 127-133
- Law, M. F., Lowy, D. R., Dvoretzky, I. and Howley, P. M. 1981. Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. Proc Natl Acad Sci U S A 78, 2727-31
- Lazary, S. 1988. Studies on Susceptibility to Equine Sarcoid. Der Praktische Tierarzt 69, 12-13.
- Lazary, S., Gerber, H., Glatt, P.A., Straub, R. 1985. Equine leukocyte antigens in sarcoid-affected horses. Equine Vet J 17, 283-286.
- Leong, CM., Doorbar, J., Nindl, I., Yoon, HS., Hibma, MH. 2010. Loss of epidermal Langerhans cells occurs in human papillomavirus alpha, gamma, and mu but not beta genus infections. J Invest Dermatol. ;130(2):472-80. doi: 10.1038/jid.2009.266.
- Lepage, M.F., Carstanjen, B., von Tscharner, C. 1998. Equine sarcoid (part I): causes, diagnosis, differential diagnosis. Praktische Tierarzt 79, 627-636.
- Lindholm, I., Murphy, J., O'Neil, BW., Campo, MS., Jarrett, WF. 1984. Papillomas of the teats and udder of cattle and their causal viruses. Vet Rec. 1;115(22):574-7.
- Lioi, MB., Barbieri, R., Borzacchiello, G., Dezzi, S., Roperto, S., Santoro, A., Russo, V., Roperto, F. 2004. Chromosome aberrations in cattle with chronic enzootic haematuria. J Comp Pathol. Aug-Oct;131(2-3):233-6.
- Literak, I., Tomita, Y., Ogawa, T., Shirasawa, H., Smid, B.L., Novotny, Adame, M.. 2006. Papillomatosis in a European bison. J. Wildl. Dis. 42, 149–153.
- Lohr, C. V., Juan-Salles, C., Rosas-Rosas, A., Paras Garcia, A., Garner, M. M., Teifke, J. P. 2005. Sarcoids in captive zebras (Equus burchellii): association with bovine papillomavirus type 1 infection. J. Zoo Wildl. Med. 36, 74–81.
- Love, A. J., Chapman, S. N., Matic, S., Noris, E., Lomonossoff, G. P., Taliansky, M. 2012. In planta production of a candidate vaccine against bovine papillomavirus type 1. Planta 236, 1305–1313
- Lucke, B., H. 1950. Ratcliff and Breedis, C. Transmissible papilloma in monkeys. Fed. Proc. 9: 337
- Lunardi, M., de Alcântara, BK., Otonel, RA., Rodrigues, WB., Alfieri, AF., Alfieri, AA. 2013b. Bovine papillomavirus type 13 DNA in equine sarcoids. J Clin Microbiol.. 51(7):2167-71. doi: 10.1128/JCM.00371-13.
- Lunardi, M., Alfieri, A.A., Otonel, R.A.A., de Alcantara, B.K., Rodrigues, W.B., de Miranda, A.B., Alfieri, A.F. 2013a. Genetic characterization of a novel bovine papillomavirus member of the deltapapillomavirus genus. Veterinary Microbiology; 162 (1), 207-213.
- Lunardi, M., Alfieri, A. A., Otonel, R. A., de Alcantara, B. K., Rodrigues, W. B., de Miranda, A. B., Alfieri, A. F. 2012. Genetic characterization of a novel bovine papillomavirus member of the Deltapapillomavirus genus. Vet. Microbiol. 162, 207–213.
- MacLachlan, N.J., Dubovi, E.J. 2011. Papillomaviridae and Polyomaviridae. In Fenner's Veterinary Virology. 4th edition. Elsevier Inc., UK, 213-223.
- Maglennon, GA., McIntosh, P., Doorbar, J. 2011. Persistence of viral DNA in the epithelial basal layer suggests a model for papillomavirus latency following immune regression. Virology. 414:153–163.
- Maglennon, GA., McIntosh, PB., Doorbar, J. 2014. Immunosuppression facilitates the reactivation of latent papillomavirus infections. J Virol.;88(1):710-6. doi: 10.1128/JVI.02589-13. Epub 2013 Oct 30. PubMed PMID: 24173230; PubMed Central PMCID: PMC3911712.
- Maran, A., Amella, C.A., Di Lorenzo, T.P., Auborn, K.J., Taichman, L.B., Steinberg, B.M., 1995. Human papillomavirus type 11 transcripts are present at low abundance in latently infected respiratory tissues. Virology 212 (2), 285–294.
- Martens, A., De Moor, A., Demeulemeester, J., Ducatelle, R. 2000. Histopathological characteristics of five clinical types of equine sarcoid. Res Vet Sci 69, 295-300.

- Martens, A., De Moor, A., Vlaminck, L., Pille, F., Steenhaut, M. 2001c. Evaluation of excision, cryosurgery and local BCG vaccination for the treatment of equine sarcoids. The Veterinary Record 149, 665-669.
- Martens, A., De Moor, A. and Ducatelle, R. (2001b) PCR detection of bovine papilloma virus DNA in superficial swabs and scrapings from equine sarcoids. Vet. J. 161, 280-286.
- Martens, A., De Moor, A., Demeulemeester, J. and Peelman, L. (2001a) Polymerase chain reaction analysis of the surgical margins of equine sarcoids for bovine papilloma virus DNA. Vet. Surg. 30, 460-467.
- Marti, E., Lazary, S., Antczak, DF., Gerber, H. 1993. Report of the first international workshop on equine sarcoid. Equine Vet J. 25(5):397-407.
- Masuda, E. K., Kommers, G. D., Martins, T. B., Barros, C. S., Piazer, J. V. 2011. Morphological factors as indicators of malignancy of squamous cell carcinomas in cattle exposed naturally to bracken fern (Pteridium aquilinum). J. Comp. Pathol. 144, 48–54.
- Matthews, K., Leong, C. M., Baxter, L., Inglis, E., Yun, K., Backstrom, B. T., Doorbar, J., Hibma, M. 2003. Depletion of Langerhans cells in human papillomavirus type 16-infected skin is associated with E6-mediated down Regulation of E-cadherin. J. Virol. 77:8378–8385.
- McBride, AA., Sakakibara, N., Stepp, WH., Jang MK. 2012. Hitchhiking on host chromatin: how papillomaviruses persist. Biochim. Biophys. Acta 1819: 820-825.
- McBride, A. A. 2008. Replication and partitioning of papillomavirus genomes. Adv. Virus Res. 72, 155–205.
- Mcentee, K. 1952. Transmissible fibropapillomas of the external genitals of cattle. Report of New York Vet. College, Cornell University. Ithaca, N.Y. P. 28. 6
- Melnick, JL. 1962. Papova virus group. Science;135: 1128-30
- Meredith, D., Elser, A. H., Wolf, B., Soma, L. R., Donawick, W. J. & Lazary, S. 1986. Equine leukocyte antigens: relationships with sarcoid tumors and laminitis in two pure breeds. Immunogenetics 23, 221–225.
- Miller, R.I., Campbell R.S.F. 1982. A survey of granulomatous and neoplastic diseases of equine skin in North Queensland. Aust Vet J 59, 33-37
- Mohammed, H.O., Rebhun, W.C., Antczak, D.F. 1992. Factors Associated with the Risk of Developing Sarcoid Tumors in Horses. Equine Vet J 24, 165-168.
- Moore, RA., Walcott, S., White, KL., Anderson, DM., Jain, S., Lloyd, A., Topley, P., Thomsen, L., Gough, GW., Stanley, MA. 2003. Therapeutic immunisation with COPV early genes by epithelial DNA delivery. Virology. 30;314(2):630-5.
- Moulton, J.E. 1954. Cutaneous papillomas on the udders of milk goats. North Am. Vet. 35: 29-33. 15.
- Munday, J.S., Knight, C.G. 2010. Amplification of feline sarcoid-associated papillomavirus DNA sequences from bovine skin. Vet Dermatol. 21 (4), 341-344.
- Munday, JS., Hanlon, EM., Howe, L., Squires, RA., French, AF. 2007. Feline cutaneous viral papilloma associated with human papillomavirus type 9. Vet Pathol. 44(6):924-7.
- Munday, JS., Knight, CG., Howe, L. 2010. The same papillomavirus is present in feline sarcoids from North America and New Zealand but not in any non-sarcoid feline samples. Journal of Veterinary Diagnostic Investigation; 22: 97–100
- Munday, JS. 2014. Bovine and human papillomaviruses: a comparative review. Vet Pathol. 51(6):1063-75. doi: 10.1177/0300985814537837.
- Munger, K., Howley, P. 2002. Human papillomavirus immortalization and transformation functions. Virus Res 89(2):213-28.
- Myers, G., Bernard, H.U., Delius, H., Favre, M., Icenogel, J., van Ranst, M.,Wheeler, C. (Eds.), 1994. Human Papillomaviruses: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos
- Narechania, A, Chen, Z, De Salle, R, Burk, RD. 2005. Phylogenetic incongruence among oncogenic genital alpha human papillomaviruses. J Virol. 79(24):15503-10.
- Nasir, L., Campo, MS. 2008. Bovine papillomaviruses: their role in the aetiology of cutaneous tumours of bovids and equids. Vet Dermatol. 19:243–254.
- Nasir, L., Gault, E., Morgan, IM., Chambers, G., Ellsmore, V., Campo, MS. 2007. Identification and functional analysis of sequence variants in the long control region and the E2 open reading frame of bovine papillomavirus type 1 isolated from equine sarcoids. Virology. 1;364(2):355-61.
- Nel, PJ., Bertschinger, H., Williams, J., Thompson, PN. 2006. Descriptive study of an outbreak of equine sarcoid in a population of Cape mountain zebra (Equus zebra zebra) in the Gariep Nature Reserve. J S Afr Vet Assoc. ;77(4):184-90.

- Nicholls, P.K., Doorbar, J., Moore, R.A., Peh, W., Anderson, D.M., Stanley, M.A. 2001. Detection of viral DNA and E4 protein in basal keratinocytes of experimental canine oral papillomavirus lesions. Virology.;284(1):82–98
- Ogawa, T., Tomita, Y., Okada, M., Shinozaki, K., Kubonoya, H., Kaiho, I., Shirasawa, H. 2004. Broad-spectrum detection of papillomaviruses in bovine teat papillomas and healthy teat skin. J. Gen. Virol. 85, 2191–2197.
- Ogawa, T., Tomita, Y., Okada, M., Shirasawa, H. 2007. Complete genome and phylogenetic position of bovine papillomavirus type 7. J. Gen. Virol. 88, 1934–1938.
- Olson, C., Cook, RH. 1951. Cutaneous sarcoma-like lesions of the horse caused by the agent of bovine papilloma. Proc Soc Exp Biol Med;77:281.
- Olson, C., Cook, R. H. 1951. Cutaneous sarcoma-like lesions of the horse caused by the agent of bovine papilloma. Proc. Soc. Exptl. Biol. & Med. 77: 281-284.
- Olson, C., Olson, RO., Hubbard-Van Stelle, S. 1992. Variations of response of cattle to experimentally induced viral papillomatosis. J Am Vet Med Assoc. 201:56–62
- Olson, C., Pamukcu, AM., Brobst, DF., Kowalczyk, T., Satter, EJ., Price, JM. 1959. A urinary bladder tumor induced by a bovine cutaneous papilloma agent. Cancer Res. Aug;19:779-82.
- Otten, N., von Tscharner, C., Lazary, S., Antczak, D.F. and Gerber, H. 1993. DNA of bovine papillomavirus type 1 and 2 in equine sarcoids: PCR detection and direct sequencing. Arch. Virol. 132, 121-131.
- Pangty, K., S. Singh, R. Goswami, G. Saikumar, and R. Somvanshi, 2010: Detection of BPV-1 and -2 and quantification of BPV-1 by real-time PCR in cutaneous warts in cattle and buffaloes. Transbound. Emerg. Dis. 57, 185–196
- Pascoe, R.R., Knottenbelt, D.C. 1999. Neoplastic conditions. In: Pascoe R.R. and Knottenbelt D.C. (editors). Manual of Equine Dermatology. Saunders, London, p. 244-252.
- Payne, JF. 1891. On the contagiousness of common warts. British Journal of Dermatology;3:185-8
- Lancet. Medical News Peculiar sign. 1849..;ii:250
- Pentertby, J. 1898. Contagious warty tumors in dogs. J. Compt. Path. & Therap. 11: 363. 13.
- Pignatari, S., Smith, E.M., Gray, S.D., Shive, C., Turek, L.P., 1992. Detection of human papillomavirus infection in diseased and nondiseased sites of the respiratory tract in recurrent respiratory papillomatosis patients by DNA hybridization. Ann. Otol. Rhinol. Laryngol. 101 (5), 408–412.
- Piscopo, S.E. 1999. The complexities of sarcoid tumors. Equine Practice 21, 14-18
- Pulley, L.T., Stannard A.A. 1990. Skin and soft tissues. In: Moulton J.E. (editors). Tumours in domestic animals. University of California Press, Los Angelos, p. 27-31.
- Ragland, W.L., Keown, G.H., Gorham, J.R. 1966. An epizootic of equine sarcoid. Nature 210, 1399
- Ragland, WL., Keown, GH., Spencer, GR. 1970. Equine sarcoid. Equine Vet J 2:2-11, Rec. 2011 Feb 19;168(7):187. doi: 10.1136/vr.c5430.
- Ragland, W.L. 1970. Equine sarcoid. Equine Veterinary Journal 2, 2-11.
- Rajcani, J. 2007. Reactivation of latent DNA viruses during immunosuppression: basic contemplation. Acta Virol. 51 (2), 77–100
- Rector, A., Lemey, P., Tachezy, R., Mostmans, S., Ghim, SJ., Van Doorslaer, K., Roelke, M., Bush, M., Montali, RJ., Joslin, J., Burk, RD., Jenson, AB., Sundberg, JP., Shapiro, B., Van Ranst, M. 2007. Ancient papillomavirus-host co-speciation in Felidae. Genome Biol.;8(4):R57.
- Rector, A., Van Ranst, M. 2013. Animal Papillomaviruses. Virology; 445 (1-2), 213-223.
- Reid, S.W.J., Gettinby, G. 1996. Statistical assessment of risk for the clinical management of equine sarcoids in a population of Equus asinus. Preventive Veterinary Medicine 26, 87-95.
- Reid, S.W.J., Gettinby, G., Fowler, J.N., Ikin P. 1994. Epidemiologic observations on sarcoids in a population of donkeys (Equus-Asinus). The Veterinary Record 134, 207-211
- Reschke, C. 2012. Successful treatment of an equine sarcoid. Case report on a combined surgical and photodynamic therapy. Tierarztl Prax Ausg G Grosstiere Nutztiere;40(5):309–13. 665–9.
- Richards, RM., Lowy, DR., Schiller, JT., Day, PM. 2006 Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. Proc Natl Acad Sci U S A 31;103(5):1522-7.
- Robles-Sikisaka, R., Rivera, R., Nollens, HH., St Leger, J., Durden, WN., Stolen, M., Burchell, J., Wellehan, JF Jr. 2012. Evidence of recombination and positive selection in cetacean papillomaviruses. Virology. Jun 5;427(2):189-97. doi: 10.1016/j.virol.2012.01.039.
- Rombaldi, R. L., Serafini, E. P., Mandelli, J., Zimmermann, E., Losquiavo, K. P. 2008. Transplacental transmission of human papillomavirus. Virol. J. 5, 106.

- Roperto, S., Borzacchiello, G., Esposito, I., Riccardi, M., Urraro, C., Lucà, R., Corteggio, A., Tatè, R., Cermola, M., Paciello, O., Roperto, F. 2012. Productive infection of bovine papillomavirus type 2 in the placenta of pregnant cows affected with urinary bladder tumors. PLoS One;7(3):e33569. doi: 10.1371/journal.pone.0033569.
- Royer, J. 1902. Des venues chez l'homme et chez les animaux. Faculte de Medecine et de Pharmacie de Lyon. Theses. 66: 67 p. 3.
- Sala A, Schuszler L, Sabau M, Luca C, Igna C, Dascalu R, Brezovan D 2010. Horse sarcoids: A morphoclinical and therapeutic experience on ten cases. Lucrari stiintifice Medicina Veterinara. 43 (2): 109-118.
- Schelhaas, M., Shah, B., Holzer, M., Blattmann, P., Kuhling, L., Day, PM., Schiller, JT., Helenius, A. 2012. Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis. PLoS Pathog, 8:e1002657.
- Schindelica, 1903. Handbuch der tierarztlichen Chirurgie and Geburtshilfe Bd. 6 Hautkrankheiten; 389.
- Schmitt, M., Fiedler, V., Muller, M. 2010. Prevalence of BPV genotypes in a German cowshed determined by a novel multiplex BPV genotyping assay. J. Virol. Methods 170, 67–72
- Schulman, FY., Krafft, AE., Janczewski, T. 2001. Feline cutaneous fibropapillomas: clinicopathologic findings and association with papillomavirus infection. Veterinary Pathology. 38: 291–6.
- Schwartz, S. 2013. Papillomavirus transcripts and posttranscriptional regulation. Virology. 445(1-2):187-96. doi: 10.1016/j.virol.2013.04.034.
- Schwartz, S., 2008. HPV-16 RNA processing. Front. Biosci. 13, 5880–5891.
- Scott, DW., Miller, WH., editors. Equine dermatology. 2nd edition. St Louis (MO): Saunders Elsevier; 2011. p. 479.
- Sennert, D. 1636. Hypomnemata physica. Frankfurt, pp 428
- Shah, SD., Doorbar, J., Goldstein, RA. 2010. Analysis of host-parasite incongruencein papillomavirus evolutionusing importance sampling. Mol Biol Evol;27:1301–14.
- Shokry, M., Naoum, E., El-Husseiny, E., Battour, A. 2015. Clinical survey of equine sarcoids in Egypt. Issues in Biological Sciences and Pharmaceutical Research Vol.3 (8),pp.86-93
- Shope, RE., Hurst, EW. 1933. Infectious papillomatosis of rabbits- With a note on the histopathology. J Exp Med. 1933 Oct 31;58(5):607-24.
- Siegsmund, M., Wayss, K., Amtmann, E. 1991. Activation of latent papillomavirus genomes by chronic mechanical irritation. J Gen Virol; 72 (Pt 11):2787–9
- Silva, MA., Altamura, G., Corteggio, A, Roperto, F., Bocaneti, F., Velescu, E., Freitas, AC., Carvalho, CC., Cavalcanti, KP., Borzacchiello, G.2013 Expression of connexin 26 and bovine papillomavirus E5 in cutaneous fibropapillomas of cattle. Vet J.;195:337–343.
- Silva, MA., Altamura, G., Corteggio, A., Roperto, F., Bocaneti, F., Velescu, E., Freitas, AC., Carvalho, CC., Cavalcanti, KP., Borzacchiello, G. 2013. Expression of connexin 26 and bovine papillomavirus E5 in cutaneous fibropapillomas of cattle. Vet J. Mar;195(3):337-43. doi: 10.1016/j.tvjl.2012.07.009.
- Silvestre, O, Borzacchiello, G., Nava, D., Iovane, G., Russo, V., Vecchio, D., D'Ausilio, F., Gault, E. A., Campo, M. S., Paciello, O. 2009. Bovine papillomavirus type 1 DNA and E5 oncoprotein expression in water buffalo fibropapillomas. Vet. Pathol. 46, 636–641.
- Singh, V., Somvanshi, R., Tiwari, A. K. 2009. Papillomatosis in Indian cattle: occurance and etiopathology. Indian J. Vet. Pathol. 33, 52–57.
- Smith, B. L. 1997. The toxicity of bracken fern (genus Pteridium) to animals and its relevance to man. In: Handbook of Plant and Fungal Toxicants, J. P. F. D'Mello, Ed., CRC Press, London, pp.63–76.
- Smith, E.M., Pignatari, S.S., Gray, S.D., Haugen, T.H., Turek, L.P. 1993. Human papillomavirus infection in papillomas and nondiseased respiratory sites of patients with recurrent respiratory papillomatosis using the polymerase chain reaction. Arch. Otolaryngol. Head Neck Surg. 119 (5), 554–557
- Spoormakers, TJ., Klein, WR., Jacobs, JJ., Van Den Ingh, TS., Koten, JW., Den Otter, W. 2003. Comparison of the efficacy of local treatment of equine sarcoids with IL-2 or cisplatin/IL-2. Cancer Immunol Immunother. Mar;52(3):179-84.
- Stadler, S., Kainzbauer, C., Haralambus, R., Brehm, W., Hainisch, E., Brandt, S. 2011. Successful treatment of equine sarcoids by topical aciclovir application. Vet Rec. 19;168(7):187. doi: 10.1136/vr.c5430.
- Stanley, MA., Moore, RA., Nicholls, PK., Santos, EB., Thomsen, L., Parry, N., Walcott, S., Gough, G. 2001. Intra-epithelial vaccination with COPV L1 DNA by particle-mediated DNA delivery protects against mucosal challenge with infectious COPV in beagle dogs. Vaccine. 6;19(20-22):2783-92.
- Stanley, MA. 2012. Epithelial cell responses to infection with human papillomavirus. Clin Microbiol Rev. .25(2):215-

22. doi: 10.1128/CMR.05028-11.

- Stocco dos Santos, R. C., Lindsey, C. J., Ferraz, O. P., Pinto, J. R., Mirandola, R. S., Benesi, F. J., Birgel, E. H., Pereira, C. A., Becak, W. 1998. Bovine papillomavirus transmission and chromosomal aberrations: an experimental model. J. Gen. Virol. 79(Pt 9), 2127–2135.
- Strafuss, AC., Smith, JE., Dennis, SM., Anthony, HD. 1973. Sarcoid in horses. Vet Med Small Anim Clin. 68(11):1246-7.
- Strauss, MJ., Shaw, EW., Bunting, H., Melnick, JL. 1949. "Crystalline" virus-like particles from skin papillomas characterized by intranuclear inclusion bodies. Proc Soc Exp Biol Med;72:46-50
- Stubenrauch, F., Laimins, L.A. 1999. Human papillomavirus life cycle: active and latent phases. Semin. Cancer Biol.;9(6):379–386.
- Studer S., Gerber V., Straub R., Brehm W., Gaillard C., Luth A., Burger D. 2007. Prevalence of hereditary diseases in three-year-old Swiss Warmblood horses. Schweizer Archiv für Tierheilkunde 149, 161-171.
- Tachezy, R., Duson, G., Rector, A., Jenson, A. B., Sundberg, J. P., Van Ranst, M. 2002. Cloning and genomic characterization of Felis domesticus papillomavirus type 1. Virology, 301, 313-321.
- Theon, A.P. 1998. Intralesional and topical chemotherapy and immunotherapy. Veterinary Clinics of North America-Equine Practice 14, 659-671
- Théon AP, Pascoe JR, Galuppo LD, Fisher PE, Griffey SM, Madigan JE. 1999. Comparison of perioperative versus postoperative intratumoral administration of cisplatin for treatment of cutaneous sarcoids and squamous cell carcinomas in horses. J Am Vet Med Assoc. 1;215(11):1655-60.
- Theon AP, Pascoe JR. 1995. Iridium-192 interstitial brachytherapy for equine periocular tumours: treatment results and prognostic factors in 115 horses. Equine Vet J;27(2):117–21.
- Torrontegui B.O., Reid S.W.J. 1994. Clinical and pathological epidemiology of the equine sarcoid in a referral population. Equine Veterinary Education 6, 85-88.
- Tsirimonaki, E., O'Neil, B. W., Williams, R., Campo, M. S. 2003. Extensive papillomatosis of the bovine upper gastrointestinal tract. J. Comp. Pathol. 129, 93–99.
- Vallvè, S.G., Alonso, A., Bravo, I.G. 2005. Papillomaviruses: different genes have different histories. Trends in Microbiology; 13 (11), 514-521.
- van Dyk, E., A. M. Bosman, E. van Wilpe, J. H. Williams, R. G. Bengis, J. van Heerden, and E. H. Venter, 2011. Detection and characterisation of papillomavirus in skin lesions of giraffe and sable antelope in South Africa. J. S. Afr. Vet. Assoc. 82, 80–85.
- Variot, G. 1894. Un cas d'inoculation experimentale des verrues de l'enfant A l'homme. J Clin Ther Inf; 2:529-34
- Varsani A, van der Walt E, Heath L, Rybicki EP, Williamson AL, Martin DP. 2006. Evidence of ancient papillomavirus recombination. J Gen Virol. 87(Pt 9):2527-31.
- Vingerhoets M., Diehl M., Gerber H., Stornetta D., Rausis C. 1988. Treatment of equine sarcoid by CO2-laser. Schweizer Archiv für Tierheilkunde 130, 113-126.
- Wile, U. J., Kingery, L. B. 1919. The etiology of common warts. Preliminary report of an experimental study. Jour. Amer. Med. Assoc. 73: 970-973
- Wilson E. On the dermopathology of Celsus. BMJ 1863; ii:446-9 5
- Wobeser, B.K., Davies, J.L., Hill, J.E., Jackson, M.L., Kidney, B.A., Mayer, M.N., Townsend, H.G. and Allen, A.L. 2010. Epidemiology of equine sarcoids in horses in western Canada. Can. Vet. J. 51, 1103-1108
- Woolford L, Rector A, Van Ranst M, Ducki A, Bennett MD, Nicholls PK, Warren KS, Swan RA, Wilcox GE, O'Hara AJ. 2007. A novel virus detected in papillomas and carcinomas of the endangered western barred bandicoot (Perameles bougainville) exhibits genomic features of both the Papillomaviridae and Polyomaviridae. J Virol. 81(24):13280-90.
- Yaguiu, A., M. L. Z. Dagli, E. H. Birgel Jr, B. C. A. A. Alves Reis, O. P. Ferraz, L. G. B. Goes, E. M. Pituco, A. C. Freitas, W. Becak, and R. C. Stocco dos Santos, 2008: Simultaneous presence of bovine papillomavirus (BPV) and bovine leukemia virus (BLV) in different bovine tissues: in situ hybidrization and cytogenetic analysis. Genet. Mol. Res. 7, 487–497.
- Zhang, P., Nouri, M., Brandsma, J.L., Iftner, T., Steinberg, B.M., 1999. Induction of E6/E7 expression in cottontail rabbit papillomavirus latency following UV activation. Virology 263 (2), 388–394.
- Zheng, Z.M., Baker, C.C., 2006. Papillomavirus genome structure, expression, and posttranscriptional regulation. Front. Biosci. 11, 2286–2302.
- Zhu, W., Dong, J., Shimizu, E., Hatama, S., Kadota, K., Goto, Y., Haga, T. 2012. Characterization of novel bovine papillomavirus type 12 (BPV-12) causing epithelial papilloma. Arch. Virol. 157, 85–91.
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II
AIM OF THE STUDY

Papillomaviridae are thought to be one of the oldest viral families that co-evolved with their hosts over millions of years. The viruses belonging to this species are claimed to be strictly species-specific and, even in experimental conditions, they do not infect any other host than their natural one, in this context BPVs belonging to *Delta* genus represent an exception for their widely documented ability to infect hosts different from cattle and in particular in horses. The present thesis focuses on the infections of BPVs in different animal species and it was aimed at expanding the actual knowledge of the biology of theses viruses.

Despite the importance of the benign and malignant tumours caused by the infection with bovine papillomavirus in cattle (Borzacchiello and Roperto, 2008), limited information is available about the distribution of the different viral types on the Italian territory. Hence, the objective of Paper I was the molecular characterization of the BPVs harbored in clinically-diagnosed papillomas of cattle. The identification of a new variant of BPV type 7 in mammary papillomas, during the above survey, allowed to determine their entire genomic sequence. Details of the main genomic features and prediction of the secondary structures of encoded proteins are provided in Paper II. In Paper III we investigated the causes of several outbreaks of a malignant proliferative skin disease that affected domestic ruminants in Sicily during 2011–2014. With the aid of a newly developed mini array kit we showed the so-called "papillomatosis" can be the result of multiple infections with epitheliotropic viruses, including zoonotic poxviruses, that cannot be properly identified with classical diagnostic techniques. The results led us to suggest a further role of BPVs during co-infections with other epitheliotropic viruses.

Paper IV is about a molecular investigation of a histologically classified fibropapilloma of a red deer shot in the Italian Alps was conducted with the objective to identify the presence of

PVs genomes. Increasing evidence suggests the commensalic role of PVs in humans and in different animal species. Consequently, in Paper V we evaluated the ability not only of *Delta* but also of the other BPVs genera to sub clinically infect species other than the host one. Specifically, we investigated the presence in healthy skin and mucosa of wildlife species of the same types detected in domestic animals. BPV types 1 and 2, give rise to sarcoids, in horses and donkeys, with E5 gene specific nucleotide substitutions. In Paper VI we investigated whether this genetic variability may be also found in BPV1 PBMC associated, of sub-clinically infected horses since viral DNA can be also found in the skin of healthy subjects and in non-sarcoid inflammatory skin conditions.

III
ARTICLES COMPENDIUM

Paper I

Short communication

Bovine papillomatosis: first detection of BPV types 6, 7, 8, 10 and 12 in Italian cattle herds

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Abstract

Limited information about the distribution of different Bovine Papillomaviruses (BPVs) types in Italy is available; therefore, this study aimed at investigate the presence of BPVs in cattle's lesions in Emilia Romagna region. Sixty four proliferative lesions were analyzed by qualitative PCR with genus and type-specific primer pairs, as well as Rolling Circle Amplification (RCA). The results showed, for the first time in Italy, the presence of BPV6, 7, 8, 10 and 12 as well as the circulation on the territory of PV types previously described in other European and extra European countries; in addition, the high prevalence of viral co-infections gives new information about viral tropism.

Keywords: Bovine papillomavirus; Co-infections; Diagnosis; Proliferative lesions; Viral types

Bovine Papillomaviruses (BPVs) induce hyperplastic generally regressing benign lesions of both cutaneous and mucosal epithelia in cattle, but the presence of environmental co- factors may also evolve into cancer. Wide incidence of BPVs, present as single or multiple infections in cattle herds in various regions of the world has been documented in literature (Schmitt et al., 2010; Carvalho et al., 2012), but limited information about the types circulating in Italy is available. In fact, the presence of the virus is mostly confirmed by clinical examination only. Our study aimed at identifying and characterizing BPVs in lesions in different anatomical sites of beef and dairy cattle from Emilia Romagna region in order to widen the knowledge about their distribution.

A total of 64 proliferative cauliflower-like lesions, clinically identified as papillomas, were collected for diagnostic purposes from head (19), vulva (1) udder and teats (44), by vet practitioners (Table 1). After DNA extraction (Qiagen, Hilden, Germany) and quality assessment by bovine β-globin gene amplification, PCR (Taq DNA polymerase Qiagen) was firstly performed with genus-specific primer pairs and consequently with the type-specific ones (Table 2). Amplification was also performed by Rolling Circle Amplification (RCA; Illustra Templiphi kit GE, Healthcare) and subsequently digested. Purified amplicons (Roche Molecular Diagnostics, Mannheim, Germany) were sequenced with Sanger method (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and chromatograms assembled (4Peaks 1.7.1).

Xi genus was identified in 50 lesions (84.7%), *Delta* in 31 (52.5%), *Dyo-Xi* in 24 (40.6%) and *Epsilon* in 2 (3.3%), while five samples tested negative for any BPV both by PCR and RCA. Co-infections with viral types belonging to three, two and one genus were detected in 8, 31 and 20 samples respectively.

According to recent findings, stating the presence of zoonotic parapoxviruses in cow clinically diagnosed with papillomatosis, we tested the five BPV negative samples and found one positive to pseudocowpoxvirus (data not shown). Parapoxviruses cause infections that fall into the category of neglected zoonosis, their presence in unspecific lesions evidence the inadequacy of the clinical diagnosis when not supported by laboratory analyses to identify zoonotic agents (Scagliarini et al., 2015).

This investigation leads to demonstrate, for the first time in Italy, the presence of BPV6, 7, 8, 10 and confirmed the presence of types1, 2, 3 and 9. The most detected types on teats and udder were BPV7 (23/44), 9 (17/44) and 10 (26/44), resembling the situation described in Japan (Hatama et al., 2008) and Brazil (Tozato et al., 2013), but also types 1, 2, 3, and 8 were identified. The latter is the most commonly detected in Germany (Schmitt et al., 2010) while in this investigation only one sample showed positivity to BPV8. BPV3 was found to be the second most frequent type in cutaneous lesions in Brazil (Carvalho et al., 2012), in our study it was identified both in the head and in udder lesions, always in association with *Delta* types. In line with other findings (Jarrett et al., 1984), we amplified BPV6 only from mammary lesions. As previously reported, BPV2 was found in anatomical regions other than bladder and it was the most frequent type (11/19) associated with head's lesions. In this site also types 1 (7/19), 3 (2/19), 7 (2/19), and 12 (2/19) were amplified.

Twenty nine samples were co-infected by two types, while three-type infections had no preferential combinations and only 20% of samples harbored a single type. It remains a subject of speculation whether the presence of one BPV type will increase the likelihood of acquiring a second one. In addition it is unknown whether the warts are caused only by one type, and the other types are only passengers or whether they are both biologically active (Schmitt et al., 2010). Nevertheless our data suggest that when different animals in the same herd suffer from papillomatosis, lesions tend to manifest with the same localization,

macroscopic effects and same or similar combination of viral types between different subjects.

None of the samples harbored types 4, 5, 11, 13 and 14. This is not surprising for BPV4, as it is frequently associated with papillomas of the upper alimentary canal (Borzacchiello et al., 2003), and BPV14 which is still an uncommon finding in cattle. On the contrary BPV5, 13 and 11 were already described in cutaneous and teat papillomas clinically resembling the ones observed in our study (Carvalho et al., 2012). Sequencing of BPVs belonging to *Xi* and *Epsilon* genera, harbored in eleven specimens, didn't allow the identification of the viral types. The only sample collected from a lesion, localized in the vulva, tested positive for type 2 but it was not possible to identify the type amplified with *Xi* genus primer pair as a noisy sequencing signal was obtained from the amplicons.

RCA amplification evidenced a limited sensitivity compared to the PCR amplification performed with genus and type specific primer pair, furthermore results were not always in accordance with PCR findings, even if in some cases RCA allowed the identification of BPV genomes where specific primers failed. In addition, in five samples RCA amplified an unknown pattern consistent with a PV genome, leading us to conclude that PCR and RCA need to be employed in parallel to increase the sensitivity when performing papillomavirus investigations.

In conclusion, our results deepen the knowledge on the presence of BPVs circulating in Emilia Romagna region and provide new information on the presence of many different viral types in Italy. To the best of the authors' knowledge, this study represents the first identification of the BPV types 6, 7, 8, and 10 in cattle in Italy, as well as BPV12 in Europe, and supports the hypothesis that these viral types are widespread. These data are considered necessary to develop proper prophylactic and therapeutic measures, based on the production

of a vaccine that may lead to a large reduction in the incidence of the diseases in animals and consequently in reduction of the economic losses caused by BPVs' infections.

Conflict of interest statement

The authors declare that there are no conflicts of interest

References

Borzacchiello, G., Ambrosio, V., Roperto, S., Poggiali, F., Tsirimonakis, E., Venuti A., Campo, M.S., Roperto, F., 2003. Bovine papillomavirus type 4 in oesophageal papillomas of cattle from the south of Italy. Journal of Comparative Pathology 128 (2-3), 203-206.

Brandt, S., Haralambus, R., Schoster, A., Kirnbauer, R., Stanek, C., 2008. Peripheral blood mononuclear cells represent a reservoir of bovine papillomavirus DNA in sarcoid-affected equines. Journal of General Virology; 89 (6), 1390-1395.

Brandt, S., Apprich, V., Hackl, V., Tober, R., Danzer, M., Kainzbauer, C., Gabriel, C., Stanek, C., Kofler, J., 2011. Prevalence of bovine papillomavirus and *Treponema* DNA in bovine digital dermatitis lesions. Veterinary Microbiology 148 (2-4), 161-167.

Carvalho, C.C.R., Batista, M.V.A., Silva, M.A.R., Balbino, V.Q., Freitas, A.C., 2012. Detection of bovine papillomavirus types, co-infection and a putative new BPV11 subtype in cattle. Transboundary and Emerging Diseases 59 (5), 441-447.

Hatama, S., Nobumoto, K., Kanno, T., 2008. Genomic and phylogenetic analysis of two novel bovine papillomaviruses, BPV-9 and BPV-10. The Journal of General Virology 89 (1), 158-163.

Jarrett, W.F., Campo, M.S., O'Neil, B.W., Laird, H.M., Coggins, L.W., 1984. A novel bovine papillomavirus (BPV-6) causing true epithelial papillomas of the mammary gland skin: a member of a proposed new BPV subgroup. Virology 136 (2), 255-264.

Scagliarini, A., Casà, G., Trentin, B., Gallina, L., Savini, F., Morent, M., Lavazza, A., Puleio, R., Buttaci, C., Cannella, V., Purpari, G., Di Marco, P., Piquemal, D., Guercio, A., 2015. Evidence of Zoonotic Poxviridae co-infections in clinically diagnosed papillomas. Journal of Veterinary Diagnostic Investigation. Dec 23

Schmitt, M., Fiedler, V., Müller, M., 2010. Prevalence of BPV genotypes in a german cowshed determined by a novel multiplex BPV genotyping assay. Journal of Virolgical Methods 170 (1-2), 67-72.

Stocco dos Santos R.C., Lindsey C.J., Ferraz O.P., Pinto J.R., Mirandola R.S., Benesi F.J., Birgel E.H., Pereira C.A.B., Beçak W. 1998. Bovine papillomavirus transmission and chromosomal aberrations: an experimental model. Journal of General Virolgy; 79 (9), 2127-2135.

Tozato C.C., Lunardi M., Alfieri A.F., Otonel R.A.A., Di Santis G.W., de Alcântara B.K., Headley S.A., Alfieri A.A., 2013. Teat papillomatosis associated with bovine papillomavirus types 6, 7, 9, and 10 in dairy cattle from Brazil. Braz. J. Microbiol.44 (3), 905-909.

Table 1- Summary of the results obtained from cutaneous and mucosal proliferative lesions collected from diseased cattle in Emilia Romagna region. Anatomical site of collection and results obtained by PCR and RCA are described. Greek letters indicate the viral genera; when in brackets, mean that it was not possible to identify the viral type.

Sample ID	**Site of collection**	**Genera identified by PCR**				**RCA results**	**BPV types detected**
		δ	***ε***	***ξ***	***Dyo-ξ***		
180	Udder					Negative	Negative
211*	Teats			X	X	Negative	10, 7
212*	Teats	X		X	X	Unknown pattern	1, 9, 7
213*	Teats					Negative	Negative
215*	Teats			X	X	BPV7	9, 7
217*	Teats	X		X	X	Negative	1, 10, 7
218*	Teats			X	X	Unknown pattern	9, 7
220*	Teats					Negative	Negative
221*	Teats	X		X	X	BPV7	2, 7, (ξ)
468	Teats			X		Negative	9, 10
469	Udder			X		BPV10	10
470	Udder			X		Negative	9, 10
471	Udder			X		BPV10	9, 10
472	Udder			X		BPV10	9, 10
473*	Udder			X		BPV10	10
474*	Udder			X		Negative	10
475	Udder		X	X		BPV9	8, 9, 10
476	Udder			X		BPV10	9, 10
477*	Udder			X	X	BPV9	9, 10, 7
478*	Udder			X		BPV10	9
479*	Udder	X		X		BPV9	2, 9
480*	Udder	X		X		BPV9	2, 9, 10
481*	Udder			X	X	BPV9	9, 10, 7
482*	Udder			X	X	BPV6	6, 9, 7
483*	Udder			X	X	BPV10	9, 10, 7
484*	Udder			X	X	BPV10	10, 7
507*	Udder			X		BPV10	9, 10
129*	Head	X				BPV1	1
254	Vulva	X		X		Negative	2, (ξ)
465	Head					Negative	Negative
466	Head			X		Negative	(ξ)
201*	Head	X		X		BPV2	2, (ξ)
202*	Head	X		X	X	Negative	1, 2, (ξ), 7
203	Head	X				Negative	1, 2
525*	Teats			X	X	Unknown pattern	10, 7
526	Teats			X	X	Negative	10, 7
527	Teats			X	X	Negative	10, 7
528*	Teats			X	X	Negative	10, 7
529	Teats	X		X	X	Negative	2, 10, 7
530*	Teats	X		X	X	Negative	2, 10, 7
531*	Teats			X	X	Unknown pattern	10, 7
532*	Teats				X	Consistent with BPV2/6	7
533*	Udder		X	X	X	Negative	(ε), 10, 7
535	Udder				X	Negative	7
536*	Teats			X	X	Negative	10, 7
130	Head	X				BPV2	2
170*	Head	X		X		BPV1	1, 3
171*	Head	X		X		BPV1	1, (ξ)
172	Head	X		X		BPV1	1, 3
173*	Head	X		X	X	BPV3	1, 3, 7
165*	Udder	X		X		BPV1	1, 3
166*	Udder	X				BPV1	1
200*	Udder					Negative	Negative
175*	Head	X		X		Negative	1, 12
176	Udder	X		X		BPV1	1, (ξ)
360*	Udder	X				BPV1	1
388*	Head	X				Negative	2
579	Udder	X		X		BPV1	1, 3
353*	Head	X		X		Unknown pattern	2, (ξ)
354*	Head	X		X		BPV2	2, 12
355*	Head	X		X		Negative	2, 3
356*	Head	X				Negative	2
357*	Head	X		X		BPV2	2, (ξ)
358*	Head	X		X		Negative	2, (ξ)

PCR= Polymerase Chain Reaction; RCA= Rolling Circle Amplification; *= multiple lesion on site of collection; BPV= Bovine Papillomavirus

Table 2 – Pimer pair used for PCR amplification of different BPV genera and types from cutaneous and mucosal proliferative lesions.

Primer name, sequence and reference	**Amplicon lenght (bp)**	**Annealing region (amplified type)**	**Annealing temperature**
BPV 310f: 5'-RACACGGATGABTTTGTYACWCGMAC-3' **BPVBPV 310r:** 5'-CCCARTGYTCHCCWTCRCARGG-3' Brandt et al., 2011	435	L1 ORF (BPV3-10)	52°C
5'B1/2-E5: 5'-CACTACCTCCTGGAATGAACATTTCC-3' **3'B1/2-E5:** 5'-CTACCTTWGGTATCACATCTGGTGG-3' Brandt et al., 2008	499 (BPV1) 497 (BPV2)	E5 ORF (BPV1, 2)	65-56°C touch down
BPV 58f: 5'-TTTTATCATGGAGAAACAGAAAGACTAC-3' **BPV BPV58r:** 5'-CAAAGCCTATRTCCATCATATCTCC-3' Brandt et al., 2011	501	L1 ORF (BPV5, 8)	63°C
BPV 3/6f: 5'-CAGTCAATTGCAACTAGATGCC-3' **BPV 3/6r:** 5'-GGCTGCTACTTTCAAAAGTGA-3' Carvalho et al., 2012	216	L1 ORF (BPV3, 6)	60°C
BPV 4f: 5'-GCTGACCTTCCAGTCTTAAT-3' **BPV 4r:** 5'-CAGTTTCAATCTCCTCTTCA-3' Stocco dos Santos et al., 1998	170	E7 ORF (BPV4)	55°C
BPV 7f: 5'-CCACCAACGCAGCCTATTGCAAG-3' **BPV 7r:** 5'-GGTGCACAAGACCTGTCTTCTTGC-3' Brandt et al., 2011	626	L2 ORF (BPV7)	57°C
BPV 9f: 5'-AAAGAGCAAATCGGGAGCACC-3' **BPV 9r:** 5'-AACTAATGACCCACTAGGGCTCC-3' Carvalho et al., 2012	264	L1 ORF (BPV9)	60°C
BPV 10f: 5'-AAGGCATTTGTGGTCTCGAGG-3' **BPV 10r:** 5'-CTAAAGAACCACTTGGAGTGCC-3' Carvalho et al., 2012	148	L1 ORF (BPV10)	60°C
BPV 12f: 5'-ATAATCCAGAGAATCAGAGACTAG-3' **BPV 12r:** 5'-GTC TTC TGG TGG TAT GCC ATC T-3' Designed in our lab	542	L1 ORF (BPV12)	53°C

PCR= Polymerase Chain Reaction; bp= base pair; ORF= Open Reading Frame; BPV= Bovine Papillomavirus.

Paper II

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Bovine papillomavirus type 7 in Italy: complete genomes and sequence variants

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Abstract

Two novel bovine papillomavirus type 7 (BPV-7) variants have been identified in teat cutaneous papillomas affecting dairy cows in northern Italy. The entire genome sequences of two BPV-7 Italian variants showed major sequence differences in the long control region (LCR) and in the L2 gene compared to the Japanese reference strain. In order to define the stability of these genetic variants, the L2 and LCR sequences of seven further BPV-7 positive isolates were characterised. An insertion of 6 amino acids in the L2 structural protein has been detected in all samples while different genetic variants have been identified for the LCR. These findings provide new insights on intra-type variability of bovine papillomaviruses and represent a starting point for future studies aimed at establishing the biological role of the different BPV genomic regions and investigating the pathogenic potential of papillomavirus variants.

Keywords: Papillomavirus; BPV-7; L2; LCR; Variants

Introduction

According to de Villiers and colleagues [1], bovine papillomaviruses (BPVs) responsible for bovine infections are classified in four genera: *Deltapapillomavirus* (BPV-1/2/13), *Epsilonpapillomavirus* (BPV-5/8), *Xipapillomavirus* (BPV-3/4/6/9/10/11/12) and *DyoXipapillomavirus* (BPV-7).

Genome organization of BPVs is similar to other papillomaviruses consisting of a single molecule of circular double-stranded DNA that encodes 8 to 10 proteins. The whole genome can be divided into three parts with different functions: a long control region (LCR) that regulates transcription and contains the origin of replication, the early (E) region that contains open reading frame (ORF) for replication, transcription and transformation and the late (L) region that contains the ORFs encoding the two structural proteins combined in the viral capsid.

Bovine papillomatosis is an infectious disease distributed worldwide among herds which causes hyperproliferative lesions of skin and mucosae. According to viral type and different environmental co-factors, BPVs can induce cutaneous papilloma (BPV-1, -2, -8), cancer of the upper gastrointestinal tract (BPV-4), cancer of the urinary bladder (BPV-1 and -2), and papillomatosis of teats and udder (BPV-1, -5, -6, -7, -9, -10, -11). Fibropapillomas and papillomas of teats and udders have important economic impact, as the local viral spread can be so extensive that cows milking may become difficult or impossible [2].

Bovine papillomavirus type 7 was originally isolated in Japan from cutaneous papillomas and healthy teat skin samples by Ogawa and colleagues [3], and it was classified in the novel *DyoX*i *Papillomaviridae* genus. Subsequently Tozato et al., [4] detected BPV-7 in Brazilian dairy herds affected by teat papillomatosis.

Limited information are available on the viral BPV types circulating in Italy, on this basis during a biomolecular screening of BPVs variety affecting cattle herds of Emilia Romagna, BPV-7 was detected for the first time. The aim of the study was to perform genomic characterisation of 9 BPV-7 samples causing teat papillomas in dairy cows.

Materials and methods

In order to evaluate the diffusion of cutaneous papillomaviruses in dairy herds from Emilia-Romagna (Italy), a molecular survey was conducted by amplifying the L1 gene using the primers FAP59/FAP64, as described by Forslund et al., [5] and subsequently sequencing the obtained 480 bp product.

DNA from nine BPV-7 positive samples was amplified by multiple-primed Rolling-Circle Amplification (RCA) using the Illustra TempliPhi 100 amplification kit (GE Healthcare, Little Chalfont, UK) following the protocol previously described by Rector et al., [6]. A digestion with restriction enzymes BamHI and Hind III was subsequently performed and products were run on a 0.8% agarose gel to confirm the presence of DNA fragments consistent with the length of a papillomaviral genome. RCA positive DNA products were precipitated in ethanol to remove salts and residual enzymes and sequenced by primer walking with an original set of primers manually designed (Supplementary table S1, Supplementary material). Sequencing was performed with an ABI Prism 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) using a DNA concentration of 800 ng per sample. Electropherograms were inspected and edited with 4Peaks 1.7.1, and contigs were obtained with SeqMan II (DNASTAR, Madison, WI, USA).

The whole genome sequences of samples 215 and 221 were deposited in the GenBank using the National Centre for Biotechnology Information (NCBI, Bethesda, MD) BankIt v3.0 submission tool (http://www3.ncbi.nlm.nih.gov/BankIt/) under accession numbers KM096428 and KM096429, respectively.

Genomes open reading frames were investigated by using the ORF Finder tool on the NCBI server of the National Institutes of Health (http://www.ncbi.nlm.nih.gov/gorf.html). Transcription factor binding sites and motifs were searched either manually or online using ScanProsite (http://www.expasy.ch/prosite).

A prediction of the secondary structures of the L2 proteins of the 215 and 221 Italian variants and BPV-7 reference strain (NC_007612) was obtained by using the PSIPRED v3.3 software of the PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/) [7], and the open resource for online prediction of protein structural and functional features PredictProtein [8].

The complete sequences of the BPV-7 215 and 221 variants were aligned with other 95 PVs at the amino acid level using ClustalW (Supplementary table S2, Supplementary material). Alignment included the concatenated regions E1, E2, L1, L2, as in García-Pérez et al., [9]. The evolutionary history of the two BPV-7 variants and the 95 further PVs was inferred by using the Maximum Likelihood method based on the LG + freqs (+F) amino acid model [10], identified as the best-suited evolutionary model for our data. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.1032)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 6.3027% sites). The analysis involved 97 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 1076 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [11].

To investigate the most variable regions of the BPV-7 genome, specific primers for the amplification of a portion of the L2 and LCR ORFs were manually designed according to the alignment of the BPV7 Italian variants 215 and 221 with NC_007612 BPV-7 (Supplementary

table S3, Supplementary material). PCR amplifications of strains 202, 217, 218, 525, 531, 532, 535 representing 7 additional BPV-7 Italian isolates, were performed in a 50 μl volume containing 20 μM of each of the appropriate primers, according to Qiagen recommendations for Taq polymerase. Cycling conditions for L2 amplification were as follows: denaturation at 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 s, 72°C for 45 s and a final extension of 72°C for 5 minutes. Cycling conditions for amplification of a portion of the LCR were as follows: 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 52°C for 30 s, 72°C for 45 s and a final extension of 72°C for 5 minutes. PCR products were purified using the High Pure PCR Product Purification Kit (Roche) following manufacturer recommendations and directly sequenced using both the forward and reverse primers. Sequencing was performed on an ABI Prism 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and sequences analysis end editing were obtained by 4Peaks 1.7.1 (Griekspoor and Groothuis, mekentosj.com)

Results

During a survey conducted to evaluate the diffusion of cutaneous papilloma in dairy herds from Emilia-Romagna (Italy), nine animals from 2 different farms were found infected with BPV-7 (Table 1).

Genomic characterisation of BPV-7 positive samples was achieved by RCA combined to BamHI and HindIII digestions, which led to the generation of a pattern consistent with the presence of a papillomavirus genome in 3 out of 9 samples (215, 218, 221). In particular BamHI produced a single fragment of approximately 8 Kb in the samples 215, 221, and 218, while digestion of samples 215 and 221 with Hind III generated four fragments of about 1 Kb, 1.3 Kb, 2 Kb and 3 Kb, consistent with the size of BPV-7 viral genome. Gel electrophoresis of HindIII digested 218-RCA products showed the presence of fragments of unexpected size, 8 Kb, 5 Kb, 3 Kb, 2,5 Kb that may be attributable to a mixture of papillomaviral DNA. Direct

sequencing of the RCA amplicons allowed to obtain clear sequences of the 215 and 221 entire genomes, while chromatograms relative to the 218 sample were very disturbed with overlapping peaks along all the sequences. ORF analysis of the 215 and 221 genome sequences showed the presence of the seven classical PV major ORFs, encoding for five early (E), two late (L) proteins, and of a LCR. Nucleotide and amino acid identities of the single ORFs with BPV-7 reference strain (GenBank n. NC_007612) are showed in Table 2. The L1 ORFs of the Italian variants showed nucleotide and amino acid identities of 99,5% and 99,8%, respectively, with BPV-7 reference strain and therefore, according to the criteria proposed from De Villiers and colleagues, [1], they can be classified as BPV-7 variants.

Complete genomes of the 215/221 BPV type 7 Italian variants consist of 7438 and 7526 bp, respectively, and nucleotide similarity with BPV-7 reference strain NC_007612 is 97,9% (215) and 98,8% (221). According to the ICTV Papillomavirus Study Group, viruses of the same type showing more than 1.0% nucleotide sequence divergence in the whole genome, should be classified as distinct variant lineages. Consequently, strains 215 and 221 can be classified as BPV type 7 variant lineages B1 and B2. The different genome length of the two BPV- 7 variants was due to in frame deletions and insertions in the L2 ORF, and in the untranslated LCR. In particular, BPV-7 221 showed an insertion of 18 nucleotides (6 amino acids, VLRPDE), at nucleotidic position 459 of the L2 ORF while the BPV-7 215 had a deletion of 108 nucleotides causing the lack of 36 amino acids in the same position. The LCRs of both 215 and 221 variants host an insertion of 156 and 118 nucleotides respectively consisting in the duplication of two sequences of 26 and 33 nucleotides respectively at the same position.

Sequence analysis of the complete genome of the Italian variants allowed to establish the presence the canonical motifs previously identified in the BPV-7 reference strain, and of 2 additional polyadenylation signals in the E1 and L2 ORFs (Table 3). The L2 amino acid

sequences of BPV-7_215 and BPV-7_221 contain most of the protein interactions domains and features conserved in all PV types, such as: Proline residues (PxxP) associated with protein-protein interaction, two highly conserved cysteine residues (C22 and C28), the consensus furin cleavage motif site (R-X-K/R-R) conserved at around amino acids 9-12 [12], and the transmembrane like domain region at its N-terminus that includes several highly conserved GxxxG motifs.

The prediction of the secondary structure of the two BPV-7 variants indicated that L2 has two alfa helix regions at the amino-terminus part of the protein followed by a different number of beta-sheets. The insertion and deletion found in the L2 proteins of the 221 and 215 BPV-7 variants fall in a region of the L2 protein predicted as a coil exposed at the surface of the protein.

Sequence variations detected within the L2 ORF and LCR of BPV-7 215 and 221 were investigated for the 7 additional BPV-7 strains found in teats papillomas. Sample 218 showed the same 108 nucleotides deletion already detected in the L2 ORF of sample 215, while the samples 202, 217, 525, 531, 532, 535 had the 18 nucleotides insertion identified in the L2 ORF of sample 221. Four variants of the LCR regions were identified. None of the 4 variants was identical to the reference sequence. Sequence of strain 202 showed 100% identity with strain 221, and 218 showed 100% identity with strain 215. Strain 217 showed an insertion of 37 nucleotides containing the same duplication of 26 nucleotide identified in variants 215 and 221 (Supplementary figure S1, Supplementary material). Samples 525, 531, 532, and 535 showed an insertion of 142 nucleotides containing three additional E2 binding sites (Supplementary figure S2, Supplementary material).

The phylogenetic trees were obtained from a concatenated E1/E2/L1/L2 amino acid alignment of isolates 221and 215 with 95 PV-types representative of the different PV genera and species (Figure 1). The resulting maximum likelihood phylogenetic trees clustered the PVs in their respective genera, according to the new PV classification [13]. Also, the main genera

associations previously observed [14; 15] were maintained. In particular, the *Delta, Epsilon,* and *Dyokappa* viruses, infecting *Artiodactyla,* grouped together with the PVs infecting *Equidae* (*Zeta, Dyoiota, Dyorho,* and EaPV1). *Phi* papillomavirus, infecting *Capra hircus* grouped with the bovine *Xi* viruses, while BPV-7 (*Dyoxi*) clustered in a heterogeneous group comprising human (*Gamma*) and animal papillomaviruses (*Pi, Tau*). As expected, the 215 and 221 strains grouped together with the reference BPV-7 in *Dyoxi* papillomaviruses. The main clusters were statistically significant and generally supported by bootstrap values greater than 80%.

Discussion

Bovine papillomatosis can be responsible of severe economic losses especially in dairy farms. Papillomas in teats and udders hamper cow milking, and can promote secondary infections leading to mastitis. To date, different papillomavirus types and genera have been identified as causative agents of papillomas affecting teats and udder [4; 16; 17; 18]

BPV-7 was firstly found in diseased and healthy teat skin samples by Ogawa and colleagues [3], who suggested its classification within a new papillomavirus genus. BPV-7 ability to induce cutaneous papillomas in teat skin was further demonstrated by Tozato and colleagues [4]. In this study, nine samples collected from teat skin lesions of dairy cows housed in two distinct farms of Emilia-Romagna region, were found to be infected with BPV-7 by PCR. Direct sequencing of the entire genome starting from RCA products was feasible only for two viral strains. This result could be due to the presence of more than one type of papillomavirus in the samples and to the presence of genomic DNA or other confounders DNA that increased background amplification [19]. RCA is based on the use of random hexamers allowing to amplify virtually all the circular DNA molecules harboured in the skin samples such as bacterial plasmids and circular DNA of bacteriophages leading to competitive inhibition of the amplification of the viral genomes and a specific electrophoresis pattern. Similarly, in this study, RCA reactions of sample 218 amplified a mixture of PV DNAs showing an ambigous

electrophoretic pattern after HindIII digestion. Seven out of 9 samples analysed in this study were found to harbour more than one type of papillomavirus, such as BPV1-2-9-10 (data not shown). Co-infections with different types of BPV have been already reported in the same lesion and in healthy skin samples [4;16 ;17; 20]. The presence of multiple BPV types in healthy or diseased skin is reminiscent of what has been observed in human skin, where different human papillomavirus (HPV) can generate co-infections and are detected frequently at very low copy numbers [21]. The phylogenetic analysis, performed on the concatenated genes E1, E2, L1, L2 of 97 viral types including the Italian BPV-7 variants, gave similar results to those obtained by Ogawa and colleagues [3] confirming that BPV-7 belongs to a novel genus whithin the family *Papillomaviridae*. The phylogenetic tree constructed with the 97 PVs' concatenated ORFs was similar to those obtained with L1 gene sequences, further confirming the reliability of PV classification based on this ORF only.

The deletions and the insertions found within the L2 and LCR ORFs of the Italian variants BPV-7_215 and 221 variants are consistent with previous findings in different papillomavirus types identified in *Arctiodactyla*. In *Cervus elaphus papillomavirus* type 1 a deletion of 14 nucleotides in the untranslated LCR region and one deletion of 9 nucleotides in the L2 ORF were identified compared to *Capreolous capreolus papillomavirus* type 1, despite to the fact that these viruses belong to the same type and genus [22]. A 129 bp deletion in E1 was detected in a BPV type 10 strain associated with a mucosal tongue papilloma in a cow, whereas previously this BPV type has been only associated with cutaneous papillomas [23]. A deleted BPV-12 circular genome (E1, E2, partial E7 and L2 ORFs) was detected in an epithelial papilloma of a bovine, together with a full-length BPV-12 genome [24]. Genomic rearrangements have been also detected in human papillomavirus and correlated to malignancy. As an example, duplications and re-arrangements in the LCR have been found in HPV-6 and -11-associated malignancies [25; 26]. Analyses of the modified LCR region, by functional assays, suggested that activity of this regulatory region was in some cases

enhanced [27] while in others, insertions and duplications did not influence the protein expression [28]. For this reason, whether the genetic BPV-7 variants possess distinct biological properties *in vivo,* remains to be determined.

The L2 and LCR ORFs of nine BPV-7 isolates have been partially sequenced in order to establish their genetic variability.

L2 is the major structural protein that composes the viral capsid, its configuration in native virions remaining partly unknown. The domains and functions of L2 appear to be well conserved across *Papillomaviridae* as the protein has a complex and multifunctional role in the biology of all PVs, notably in virion assembly and early events of infection [29].

All the L2 ORFs sequenced showed an insertion of 18 nucleotides compared to the L2 of the BPV-7 reference strain suggesting that this sequence variant is stable and frequent in the Italian BPV-7 variants. The deletion of 36 amino acids was detected only in samples 218 and 215, collected in the same farm, leading to speculate that these BPV-7 isolates represent abortive infections despite to the fact that both animals showed clinical lesions. On the other hand 218 and 215 samples were found co-infected with other BPV types that could be the real causative agents of the disease. The deletion is in fact located in a predicted loop of the L2 protein. To a certain extent, loops are known to be flexible secondary structures that can tolerate variation, but in most of the cases, for instance when catalytically active residues are present in these structures or when they adopt important positions for protein fold, a deletion could interact with the structural and functional role of the protein [30]. Further studies with virus-like particles (VLPs) and pseudovirion assays will be necessary to verify if the deletion and insertion detected in the L2 BPV-7 variants may affect viral entry and DNA encapsidation.

Currently there aren't commercial VLP-based vaccines for use in animals. Vaccination studies in animal challenge models with L2 or part of it produced in bacteria, showed the production of neutralizing antibodies for a wide spectrum of PV types [31; 32; 33] for this

reason L2 has been considered a good candidate to constitute a PV vaccine in animals. Moreover, the N-terminal of the L2 protein is highly conseved in all PVs type and includes regions that are recognized by neutralizing antibody [34; 29]. Our findings evidenced intra-type variability of BPV-7 L2, and further studies on variants will clarify the consequences of the amino acid deletions on the antigenicity and will add information on functional motifs of this PV structural protein.

Less conserved regions of the genome, such as the LCR region, are useful to identify genetic variants and are therefore considered informative also for phylogeographic analysis [35]. The LCR regions of PV contains the origin of replication and enhancer elements that are responsive to cellular factors as well as to virally encoded transcriptional regulatory proteins like E2, which is also required for DNA replication. This region plays an important role in the pathogenesis and transformation activity of PV because it functions as the transcriptional control unit of the virus. Previous studies have shown that intra-type sequence variation within the LCR is frequent and, in some cases, it alters the transcriptional activity, and may in turn influence pathogenesis and transformation [36; 35]. In our study we found 4 different LCR sequence variants. Genomic modifications were independent from the site of isolation and the features of the lesions which was apparently similar in all samples. The variant carrying an insertion of 142 nucleotides, leading to 3 predicted additional E2 binding sites, was the most frequent (4/9 samples), this may suggest an enhanced transcription activity in insertion's carriers.

Conclusion

For detailed and refined scenarios of PV diversification, increasing insights into the representativeness of each PV type are needed to better understand their evolution [37]. Despite the exponential increase of completely characterized animal papillomavirus genomes observed over the last decades, very little is known about intra-type variability, as a single

genome for each animal papillomavirus type is usually sequenced. In fact, differently from HPV, there is a limited availability of sequenced non-human PV types, subtype and variants. In light of this, the data obtained from this study further contributes to a better understanding on intra-genus variability of bovine papillomaviruses. The biological significance of the L2 and LCR insertions and deletions, identified in the Italian BPV-7 variants, need to be further investigated.

Compliance with ethical standard

Authors declare that they have no conflict of interest. All applicable international and national guidelines for the care and use of animals were followed collecting samples analysed in this study.

REFERENCES

1. E.M. de Villiers, C. Fauquet, , T.R. Broker, H.U. Bernard, H. zur Hausen, Virology. 324, 17-27 (2004)
2. G. Borzacchiello, F. Roperto, Vet. Res. 39,45 (2008)
3. T. Ogawa, Y. Tomita, M. Okada, H. Shirasawa, J. Gen. Virol. 88, 1934-1938 (2007)
4. C.C. Tozato, M. Lunardi, A.F. Alfieri, R.A. Otonel, G.W. Di Santis, B.K. de Alcântara, S.A. Headley, A.A. Alfieri, Braz. J. Microbiol. 44, 905-9 (2013)
5. O. Forslund, A. Antonsson, P. Nordin, B. Stenquist, B.G. Hansson, J. Gen. Virol. 80, 2437-43 (1999)
6. A. Rector, G.D. Bossart, S.J. Ghim, J.P Sundberg, A.B. Jenson, M. Van Ranst, J. Virol. 78, 12698-702 (2004)
7. D.W.A. Buchan, F. Minneci, T.C.O. Nugent, K. Bryson, D.T. Jones, Nucleic Acids Res. 41 (W1): W340-W348 (2013)
8. G. Yachdav, E. Kloppmann, L. Kajan, M. Hecht, T. Goldberg, T. Hamp, P. Hönigschmid, A. Schafferhans, M. Roos, M. Bernhofer, L. Richter, H. Ashkenazy, M. Punta, A. Schlessinger, Y. Bromberg, R. Schneider, G. Vriend, C. Sander, N. Ben-Tal, B. Rost, Nucleic Acids Res. 42 (Web Server issue):W337-43 (2014)
9. R. García-Pérez, C. Ibáñez, J.M. Godínez, N. Aréchiga, I. Garin, G. Pérez-Suárez, O. de Paz, J. Juste, J.E. Echevarría, I.G. Bravo, Genome Biol. Evol. 6, 94-104 (2014)
10. S.Q. Le, O. Gascuel, Mol. Biol. Evol. 25,1307-20 (2008)
11. K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, Mol. Biol. Evol. 30, 2725-2729 (2013)
12. R.M. Richards, D.R. Lowy, J.T. Schiller, P.M. Day. Proc Natl Acad Sci U S A 103(5):1522-7 (2006)
13. H.U. Bernard, R.D. Burk, Z. Chen, K. Van Doorslaer, H. Hausen, E.M. De Villiers, Virology. 401, 70-79 (2010)
14. M. Gottschling, M. Göker, A. Stamatakis, O.R. Bininda-Emonds, I. Nindl, I.G. Bravo, Mol. Biol. Evol. 8, 2101-13 (2011)

15. R. Lecis, G. Tore, A. Scagliarini, E. Antuofermo, C. Dedola, C. Cacciotto, G.M. Dore, E. Coradduzza, L. Gallina, M. Battilani, A.G. Anfossi, M. Muzzeddu, B. Chessa, M. Pittau, A. Alberti, Vet. Microbiol. 170, 213-23 (2014)
16. T. Ogawa, Y. Tomita, M. Okada, K. Shinozaki, H. Kubonoya, I. Kaiho, H. Shirasawa, J. Gen. Virol. 85, 2191-7 (2004)
17. Y. Maeda, T. Shibahara, Y. Wada, K. Kadota, T. Kanno, I. Uchida, S. Hatama, Vet. Microbiol. 121, 242–248 (2007).
18. S. Hatama, , K. Nobumoto, T. Kanno, J. Gen. Virol. 89, 158-63 (2008)
19. Y. Marincevic-Zuniga, I. Gustavsson, U. Gyllensten, Virology. 432, 57-62 (2012)
20. M. Schmitt, V. Fiedler, M. Müller, J. Virol. Methods. 170, 67-72 (2010)
21. A. Antonsson, O. Forslund, H. Ekberg, G. Sterner, B.G. Hansson, J. Virol. 74,11636-41 (2000)
22. A. Scagliarini, L. Gallina, M. Battilani, F. Turrini, F. Savini, A. Lavazza, M. Chiari, E. Coradduzza, A. Peli, K. Erdélyi, A. Alberti, Vet. Microbiol. 30, 252-259 (2013)
23. W. Zhu, J. Dong, K. Uchida, K. Watanabe, H. Nakayama, Y. Goto, T. Haga, Vet. J. 199, 303-305 (2014)
24. J. Dong, W. Zhu, Y. Goto, T. Haga, J. Vet. Med. Sci. 75,179-82 (2013)
25. M.T. Chin, T.R. Broker, L.T. Chow, J. Virol. 63, 2967-76 (1989)
26. A. Rübben, S. Beaudenon, M. Favre, W. Schmitz, B. Spelten, E.I. Grussendorf-Conen, J. Gen. Virol. 73, 3147-3153 (1992)
27. T.C. Wu, P. Mounts, J. Virol. 62, 4722-4729 (1988)
28. C.E. Combrinck, R.Y. Seedat, C. Randall, Y. Roodt, F.J. Burt, Epidemiol. Infect. 140, 1095-101(2012)
29. J.W. Wang, R.B. Roden, 445, 175-86 (2013)
30. M.V.A Batista, T.A.E Ferreira, A.C. Freitas, V.Q. Balbino, Infect. Genet. Evol. 11, 2026–2033 (2011)
31. M.S. Campo, B.W. O'Neil, G.J. Grindlay, F. Curtis, G. Knowles, L. Chandrachud, Virology. 234, 261–266 (1997)
32. R. Gambhira, S. Jagu, B. Karanam, P.E. Gravitt, T.D. Culp, N.D. Christensen, R.B. Roden, J.Virol. 81, 11585–11592 (2007)
33. S. Jagu, B. Karanam, R. Gambhira, S.V. Chivukula, R.J. Chaganti, D.R. Lowy, J.T. Schiller, R.B.S. Roden, J. Natl. Cancer Inst. 101, 782–792 (2009)
34. I. Rubio, H. Seitz, E. Canali, P. Sehr, A. Bolchi, M. Tommasino, S. Ottonello, M. Muller, Virology. 409, 348–359 (2011)
35. Trewby, H., Ayele, G., Borzacchiello, G., Brandt, S., Campo, M.S., Del Fava, C., J. Marais, L. Leonardi, B. Vanselow, R. Biek, L. Nasir, Gen. Virol. 95, 2748-2756 (2014)
36. L. Nasir, E. Gault, I.M. Morgan, G. Chambers, V. Ellsmore, MS. Campo, Virology 364, 355-361. (2007)
37. I.G. Bravo, M. Félez-Sánchez, Evol. Med. Public Health. 28, 32-51 (2015)

Table 1: Samples collected and sequenced during this study

sample n°	**Farm**	**sequence**
202 (GenBank KU351286 and KU351279)	Farm1	portion of L2 and URR sequenced
215 (GenBank KM096428)	Farm 2	complete genome sequenced
217 (GenBank KU351287 and KU351280)	Farm 2	portion of L2 and URR sequenced
218 (GenBank KU351288 and KU351281)	Farm 2	portion of L2 and URR sequenced
221 (GenBank KM096429)	Farm 2	complete genome sequenced
525 (GenBank KU351289 and KU351282)	Farm1	portion of L2 and URR sequenced
531 (GenBank KU351290 and KU351283)	Farm1	portion of L2 and URR sequenced
532 (GenBank KU351291 and KU351284)	Farm1	portion of L2 and URR sequenced
535 (GenBank KU351292 and KU351285)	Farm1	portion of L2 and URR sequenced

Table 2 Predicted amino acid and nucleotide features of the three BPV-7 viruses

	BPV7_215 KM096428	**BPV7_221 KM096429**	**BPV7 NC_007612**
genome size	7438bp	7526bp	7412bp
GC content	45,71%	48,88%	45,53%
polyadenylation signal (AATAAA)	1369-1374 (E1) 4056-4061 (L2) 5250-5255 (L2) 5802-5807 (L1) 6981-6986 (LCR) 7373-7378 (LCR)	1369-1374 (E1) 4056-4061 (L2) 5376-5381 (L2) 5928-5933 (L1) 7107-7112 (LCR) 7456-7461 (LCR)	4077-4082 (L2) 5931-5936 (L1) 7110-7115 (LCR) 7342-7347 (LCR)
E2 binding sites ACC(N5-7)GGT	45-56 (LCR) 3118-3129 (E2) 7000-7011 (LCR) 7041-7053 (LCR) 7407-7417 (LCR)	45-56 (LCR) 3118-3129 (E2) 7126-7137 (LCR) 7167-7179 (LCR) 7495-7505 (LCR)	45-56 (LCR) 68-81 (LCR) 3118-3129 (E2) 7129-7140 (LCR) 7170-7182 (LCR) 7215-7226 (LCR) 7381-7391 (LCR)
binding site for nuclear factor 1 (TTGGCA)	1682-1687 (E1) 3001-3006 (E2) 3519-3524 (E2) 4824-4829 (L2) 6022-6027 (L1) 7097-7102 (LCR)	1682-1687 (E1) 3001-3006 (E2) 3519-3524 (E2) 4950-4955 (L2) 6148-6154 (L1) 7223-7228 (LCR)	1682-1687 (E1) 3001-3006 (E2) 3519-3524 (E2) 4953-4958 (L2) 6151-6156 (L1\) 7226-7231 (LCR)
	Predicted amino acid features		
TATA box like sequence	57-63 (LCR)	57-63 (LCR)	57-63 (LCR)
zinc binding domain in E6 CxxC(x29)CxxC	99-135	99-135	99-135
LxCxE in E7	not present	not present	not present
zinc binding domain in E7 CxxC(x32)CxxC in E7	59-98	59-98	59-98

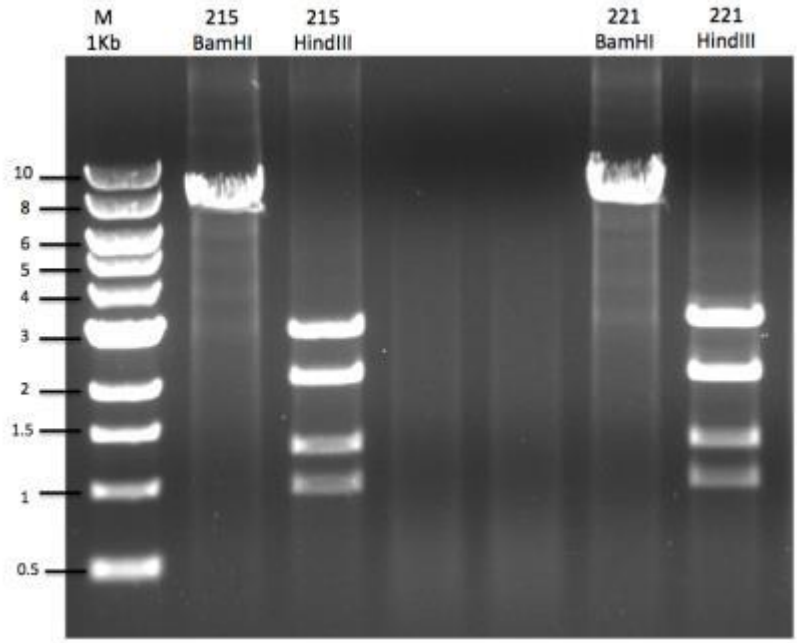
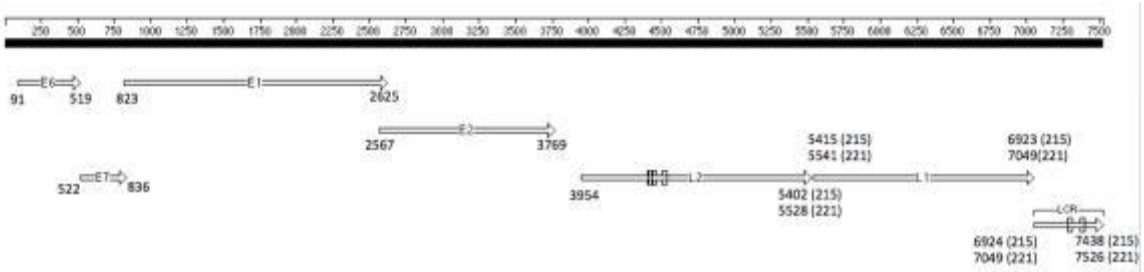


Fig. 1 Electrophoresis of RCA products after BamHI and HindIII digestion. Lane M 1 kb DNA ladder (New England Biolabs). BamHI produced a single fragment of approximately 8 kb and HindIII generated four fragments of about 1, 1.3, 2 and 3 kb in both samples 215 and 221.



ORF	BPV7_215_KM096428 (aa)	BPV7_221_KM096429 (aa)
E6	99,5 (98.6)	99,5 (98.6)
E7	99,4 (99)	99,4 (99)
E1	99,3 (99,2)	99,3 (99,2)
E2	98 (97,8)	98,2 (98)
E4	98 (94.1)	99,3 (96.1)
L2	98,2 (97,9)	98,2 (97,9)
L1	99,5 (99,8)	99,5 (99,8)
URR	96,2	96
entire genome	**97,9**	**98,8**

Fig. 2 Genomic map of the BPV-7 Italian variants 215 and 221. The ORFs are depicted as rectangle. The deletion/insertion compared to the reference BPV-7 strain are indicated as vertical lines inside the rectangles. Arabic numerals indicate genome's nucleotide positions. The table shows the nucleotide and amino acid (aa) percent identities between the BPV-7 reference virus and the new BPV-7 variants.

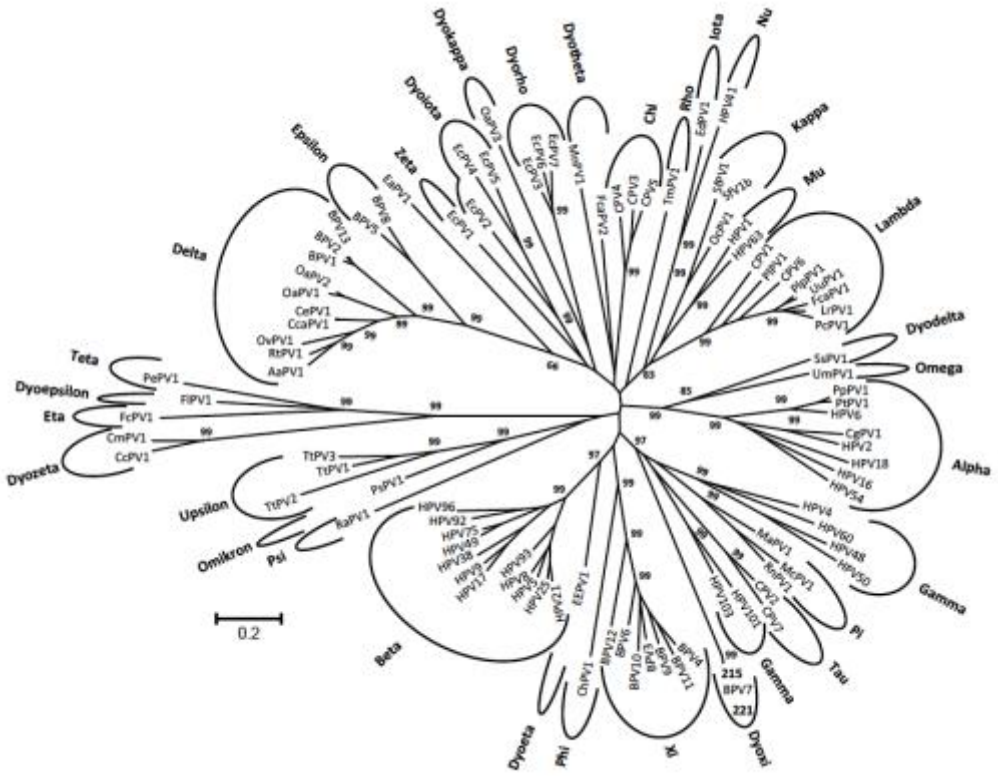


Fig. 3: Well-resolved Maximum Likelihood tree including BPV-7_215, BPV-7_221 and 95 PV-types representative of the different papillomavirus genera and species. ML tree of the 95 PVs was inferred by using a combined E1–E2–L1-L2 amino acid sequence analysis. PV genera are indicated in Greek letters and according to the PAVE website (http://pave.niaid.nih.gov). Branch lengths are drawn to scale. Numbers on branches are ML bootstrap support values. Only values above 70 are shown.

Paper III

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Evidence of Zoonotic Poxviridae co-infections in clinically diagnosed "papillomas" using a newly developed mini-array test.

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Abstract

A mini-array test, for the rapid detection of poxviruses in animals and humans, was developed within the FP7 POC4PETS project. The method is based on detection that combines target nucleic acid amplification by PCR and specific hybridization, using enzyme-linked antibodies, allowing the multiple identification of zoonotic orthopoxviruses and parapoxviruses in animals and humans' biological samples. With 100% specificity, the test rules out the possibility of cross-reactions with viral agents causing look-alike diseases. The assay was employed on the field to investigate the causes of several outbreaks of a malignant

proliferative skin disease that affected domestic ruminants in Sicily during 2011-2014. Due to the aspect of the lesions, the animals were clinically diagnosed with papillomatosis. The mini-array test allowed the identification of co-infections caused by more than one viral species belonging to the Parapoxvirus and Orthopoxvirus genera, either in goats or in cattle. The study suggests that the so-called "papillomatosis" can be the result of multiple infections with epitheliotropic viruses including zoonotic poxviruses that cannot be properly identified with classical diagnostic techniques.

Keywords: diagnosis; epitheliotropic virus; mini-array test; papilloma; Parapoxvirus; Orthopoxvirus

The families Papillomaviridae and Poxviridae include a number of viral species affecting different mammalians. The family Papillomaviridae (PV) now comprises 32 genera[21] several of which infect ruminants. In ruminant species, papillomaviruses induce hyperplastic benign lesions of both cutaneous and mucosal epithelia that generally regress, but may also occasionally persist with the possibility to evolve into cancer in the presence of environmental carcinogenic co-factors. In the Poxviridae family, the genera Parapoxvirus (PPV) and Orthopoxvirus (OPV) include viruses that cause skin lesions in domestic and wild ruminants as well as in humans. The parapoxviruses: Orf virus (OV), Bovine papular stomatitis virus (BPSV), Pseudocowpoxvirus (PCPV) and the orthopoxviruses: Vaccinia virus (VACV) Cowpoxvirus (CPXV) infect via damaged skin and give rise to proliferative and/or ulcerative lesions of the skin and mucosae. Papillomavirus and poxvirus infections recognize the same risk factors in fact, being caused by highly resistant agents, they do not necessarily need a direct contact between infected and susceptible host to be transmitted. The shed scabs, or necrotic skin or mucosal tissue, contain high titres of stable viruses which can remain infectious long enough to sustain indirect transmission.

Both papillomaviruses and poxviruses infections in animals are usually diagnosed symptomatically and only on occasion a laboratory confirmation is requested. In fact, the clinical signs are considered typical, however the lesions can be easily confused with those shown by other diseases of ruminants such as those caused by FMDV, BTV, SPXV, CPXV. Early recognition of the etiologic agents allows proper control measures and disease management strategies, to prevent diseases spread among animals and humans in case of zoonotic viruses. At present, there are no routine diagnostic tests for poxviruses and this can lead to the possibility of mis-diagnosis with other epitheliotropic pathogens[14]. Poxvirus zoonoses fall into the category of neglected zoonoses, and their incidence in animals and humans is often unknown or greatly underestimated. In humans, under-reporting leads to underestimating the true number of DALYs (Disability-adjusted life year), which can be averted by an effective control. The availability of highly specific and sensitive diagnostic assays, is considered critical to perform an accurate differential diagnosis and to understand the real incidence and prevalence of zoonotic poxviruses among mammals[14]. The aim of the study was to identify, through classical and innovative diagnostic methods, the viruses responsible for a number of outbreaks of a disease characterized by massive cutaneous lesions in domestic ruminants in Sicily. The clinical diagnosis was generically defined as "papillomatosis" since the animals were showing multiple tumour-like lesions disseminated on the entire body (Fig.1a). In many cases, the symptoms were characteristic of a systemic disease with anorexia, lethargy and death, due to starvation, in the most severely affected animals. Pathological samples (Table 1) consisting in scabs and proliferative lesions, were submitted for standard histopathological examinations and negative staining electron microscopy[12]. Histopathology mainly identified hyperkeratosis and acanthosis of the epidermis and proliferation of the fibroblasts, consistent with fibropapillomatosis, in cows, while degenerative changes in the stratus spinosum, epidermal hyperplasia, hyperkeratosis, ballooning and degeneration of keratinocytes with eosinophilic inclusion bodies, were mainly

evidenced in the lesions of sheep and goats (Fig. 1b, c). Negative staining Electron Microscopy (nsEM) leaded to the identification of Papillomavirus particles in the samples collected from diseased cows and Parapoxvirus virions in affected small ruminants. Viral DNA was extracted from the pathological specimensa and analysed by rolling circle amplifications[b] followed by restriction enzyme digestions, as previously described19, to characterize the papillomavirus (BPV) circular DNA. Different sets of primers3,4 have been used to further characterise the identified PV types. The DNA samples, purified from the lesions of all the diseased ruminants, were further analysed using a newly developed mini-array assay developed within the FP7 POC4PETS project. This assay allows the identification of different zoonotic poxviruses, belonging to the genera Parapoxvirus and Orthopoxvirus, within the same sample. The test is based on a detection approach that combines target nucleic acid amplification by PCR and specific hybridization to probes spotted on a mini-array. The assay, versus standard PCRs, showed 100% specificity, 87% sensitivity and a limit of detection of 1 fg of viral DNA. The mini-array has numerous advantages over other molecular diagnostic tools such as a straightforward interpretation, allowed through one step multiple-detection of the hybrids, which yields coloured spots on the mini-array, visible to the naked eye. The principles of the test are briefly described here: for the multiple detection of orthopoxviruses and parapoxviruses, two target genes: HA, encoding the haemagglutinin, and B2L, encoding the major envelope protein, have been selected for OPV and PPV respectively. The cytochrome C oxidase subunit I (COX I) gene, of different animal species, including sheep, goat, cow, horse, dog, cat and human, was also selected as internal control. Oligonucleotide primers for targets and probes amplifications, were designed using Primer3Plus[c] web tool[25] and OligoAnalyzer[d] 3.1 software. To allow multiplex amplifications, for each target gene the best primers candidates for probes and targets have been defined as well as the optimal PCR conditions (based on amplicon yield, specificity, sensibility, no false positive). Specific probes of OPV, PPV and positive control were obtained by PCR

amplification with FastStart Taq DNA Polymerase[e] .Mini-arrays were then generated by dotting the double-stranded DNA probes onto a positively charged nylon membranef using an arrayerg and denaturing the DNA probes by incubation of the membrane in NaOH solution. Then, membrane was prehybridized in hybridization buffer containing 1% skimmed milk and dried. Targets are thus labelled during multiplex PCR by incorporating digoxigenin-11-dUTP[h] with FastStart Taq DNA Polymerase. The subsequent detection assay consists of a target-probe hybridisation step followed by a colorimetric revelation of the hybrids using enzyme-conjugated anti-digoxigenin antibody[i], producing a dark blue precipitate in presence of appropriate substrate, (Fig. 2). The mini-array analyses, performed on the DNA purified from all the diseased ruminants, lead us to confirm that all the sheep and goats were infected with PPV. Seven out of twelve cows, previously found positive for different BPV types using RCA analysis, were also found co-infected with parapoxviruses (Table 1). Surprisingly, two goats from Messina Province and two cows from Ragusa Province, showed a co-infection with Parapoxvirus and Orthopoxvirus (Table 1). To further characterise the poxviruses identified by the mini-array test, the viral DNA was subsequently amplifiedj by PPPI and PPPIV primers targeting the Parapoxvirus B2L gene as previously described[15]. PCR amplification of the OPV's DNA was performed using the primers rpo f2 5' ATG TCG AGC TTT GTT ACC AAT 3' and rpo r2 5' TGC CAT AGT ATT CGT GTT TATACT 3'[l] targeting the highly conserved Rpo 18 gene that encodes the DNA dependent RNA polymerase of Orthopoxviruses (OPV)[2]. Thermal cycler conditions were set with an initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72 °C for 45 seconds and a final extension at 72°C for 10 minutes. The PCR, performed on the mini-array positive samples, lead to the amplification of specific fragments of 495 bp and 526 bp respectively for the B2L gene of PPVs and Rpo 18 gene of OPVs. The amplified products were subsequently purifiedk and sequenced[l]. Sequence analysis of the PCR products lead us to identify BPSV in three cows and PCPV in four

animals, while sheep and goats were shown to be all infected with OV. The Rpo 18 nucleotide sequences were aligned using ClustalWm with previously published OPV sequences from GenBank, showing an identity ranging from 97.3% to 100% among the four OPVs detected in the Sicilian outbreaks. The alignments performed, with published OPV sequences, showed a nucleotide identity ranging from 99.6% to 100% between 9605-425, 14647-429, 33225-498 and CPXV strain UK2000_K2984 (id. HQ420900). At the amino acid level, a complete identity was detected with multiple CPXV and VACV strains. A similarity of 98.1% and 98.8%, at nucleotide and amino acid level respectively, was detected between the strain 32063-501 and CPXV EleGri07/01 (id. KC813507). Phylogenetic trees were generated by the neighbour-joining method21 with 1000 bootstrap replicates10 using the Tamura 3-parameter model[22,23,n]. These analyses evidenced that the four OPV strains cluster into two distinct groups (Fig. 3). To determine the viability of viruses in samples, 60% confluent Vero monolayers were inoculated with 10-fold dilutions of sonicated tissue extract6. Infected cells were incubated at 37°C in a 5% CO2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM). Cell infection was monitored microscopically by observation of OPV-specific cytopathic effect (CPE). In order to quantify the OPV DNA in the pathological samples, a Real time SYBR Green PCR was performed[j,o]. The absolute quantification of the viral DNA has been obtained by plotting 10-fold dilutions (1.7x101 to 1.7x105 copies/µL) of pUC19 plasmid containing the cloned target fragment. No CPE was observed in inoculated cell culture while the quantitative PCR revealed an OPV load ranging from 10^1 to 10^3 DNA copies/µL, suggesting that DNA positive samples might not contain viable virus. In conclusion, we investigated the aetiological agents responsible for severe cutaneous cases of diseases in domestic ruminants using classical diagnostic techniques and an innovative molecular probe-based method able to simultaneously identify different zoonotic poxviruses within the same sample. Our data lead us to demonstrate that classical techniques, such as histopathology and electron microscopy, may not be sufficiently effective and reliable for the

diagnosis. The mini-array assay used in this study revealed that some of the animals affected by severe proliferative lesions, clinically defined as "papillomas", were in fact co-infected with zoonotic PCPV, BPSV and OPV. It is important to emphasize that zoonotic poxviruses share risk factors and clinical features with other life-threatening diseases in humans18, for this reason, rapid and reliable diagnostic methods, like the one used in this study, have the potential to allow the rapid assessment of potentially fatal infections in humans. With the aid of this new method, it was possible to demonstrate that two goats and two cows, from different farms, were co-infected with different epitheliotropic viruses. In particular, the cattle identified as 14647-429 and 33225-498, that showed the presence of papillomavirus particles by electron microscopy, were also co-infected with zoonotic poxviruses: BPSV and PCPV respectively and a virus belonging to Orthopoxvirus genus. Sant'Ana and colleagues (2013) already reported co-infections of Parapoxvirus and Orthopoxvirus in cattle in Brazil9. The description of the clinical cases seen in Brazil, were consistent with those found in the Italian outbreaks. Despite the large number of diseased animals, the authors were able to demonstrate the simultaneous presence of Parapoxvirus and Orthopoxvirus particles, by electron microscopy in the pathological sample of only one animal and, as in our study, no CPE was detected in inoculated cell cultures. Further trials, using alternative cell substrates, will be necessary to rule out the possibly that the pathological samples contained only DNA remnants or viral particles with compromised integrity by either the animal immune system or by external factors during the sample collection or processing [11]. These results indicate that tissue culture isolation is less sensitive than molecular methods to detect zoonotic poxvirus in co-infected animals while the mini-array method shows to be reliable, robust and suitable to be applied in areas where OPV and PPV co-circulate.

To our knowledge, this is the first report of OPV infections in domestic ruminants in Italy. Co-infections with Orf virus-like parapoxvirus and VACV were already reported in dairy cow9 while we identified the simultaneous presence of Orf virus and OPV in two goats.

Recently, co-infections with VACV and PCPV in a rural worker and in dairy cow have also been described1. This study further proves that co-infections with OPV and PPVs may occur in cattle not only involving PCPV but also BPSV. The high sequence identity and the circulation of CPXV in Italy in different animal species [5,6,7] lead us to conclude that the OPV DNA, detected in domestic ruminants might be CPXV. This virus used to be enzootic in cattle, however such infections were not diagnosed over the last decades, while individual cases of cowpox have been repeatedly found in cats or exotic zoo animals that were responsible for human transmission[8,13,16,17,24,26]. It is not possible to conclude that CPXV was the causal agent of the lesions reported in domestic ruminants, in fact its low genomic load can be the result of the interference caused by multiple viral agents replicating at the same time in the same target cell. On the other hand, the presence of CPXV DNA, in the pathological samples of domestic ruminants, lead us to speculate that the virus may use these animal species as reservoirs to spread and persist in the environment through the scab material produced by other epitheliotropic viruses.

Sources and Manufacturers

a. NucleoSpin® Tissue Kit, Macherey Nagel GmbH & Co. KG, Düren, Germany
b. Templiphi 100, GE Healthcare, Milano, Italy
c. Primer3Plus, bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/
d. OligoAnalyzer 3.1 web tool (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/), Integrated DNA Technologies, Inc, Coralville, Iowa, USA
e. FastStart Taq DNA Polymerase, Roche diagnostics GmbH, Mannheim, Germany
f. Hybond-N, Amersham
g. Biomek 2000, Beckman Coulter
h. digoxigenin-11-dUTP, Roche Diagnostics GmbH, Mannheim, Germany
i. Anti-Digoxigenin-POD, Fab fragments, Roche Diagnostics GmbH, Mannheim, Germany
j. PCR reagents, Qiagen, Germany
k. High Pure PCR Product purification kit, Roche Molecular Diagnostics, Mannheim, Germany
l. ABI PRISM 3730, Applied Biosystems, Foster City CA, USA
m. DNASTAR, Madison WI, USA
n. MEGA 6 software, Center for Evolutionary Medicine and Informatics, Tempe, USA.
o. Rotor Gene 3000, Corbett Research, Sydney, AU

Conflicts of Interest

The authors declare no potential conflict of interest with respect to the research, authorship and publication of this article.

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References

1. Abrahão JS, Silva-Fernandes, AT, Assis, FL, et al.: 2010, Human Vaccinia virus and Pseudocowpox virus co-infection: Clinical description and phylogenetic characterization. J Clin Virol 48:69–72.

2. Ahn BY, Jones EV, Moss B: 1990, Identification of the vaccinia virus gene encoding an 18-kilodalton subunit of RNA polymerase and demonstration of a 5_poly(A) leader on its early transcript. J Virol 64:3019–3024.

3. Brandt S, Apprich V, Hackl V, et al.: 2011, Prevalence of bovine papillomavirus and Treponema DNA in bovine digital dermatitis lesions. J Vet Microbiol 148:161-167.

4. Brandt S, Haralambus R, Schoster A, et al.: 2008. Peripheral blood mononuclear cells represent a reservoir of bovine papillomavirus DNA in sarcoid-affected equines. J Gen Virol 89:1390-1395.

5. Canese MG, Lavazza, A, Massone A, et al.: 1997, Feline poxvirus infection. A case report. Schweiz Arch Tierh: 139:454-457.

6. Cardeti G, Brozzi A, Eleni C, et al.: 2011, Cowpox virus in Llama, Italy. Emerg Inf Dis: 17: 237 1513-1515.

7. Carletti F, Bordi L, Castilletti C, et al.: 2009, Cat-to-human orthopoxvirus transmission, northeastern Italy. Emerg Inf Dis: 15:499–500.

8. Dabrowski PW. Radonić A. Kurth A, Nitsche A.: 2013. Genome-wide comparison of cowpox viruses reveals a new clade related to Variola virus. PLoS One: 8(12), e79953.

9. de Sant'Ana FJF, Leal FAA, Rabelo RE, et al.: 2013, Coinfection by Vaccinia virus and an Orf virus–like parapoxvirus in an outbreak of vesicular disease in dairy cows in Midwestern Brazil. J Vet Diagn Inv: 25:267-272.

10. Felsenstein J: 1985, Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

11. Gallardo-Romero NF, Velasco-Villa A, Weiss SL, et al.: 2011, Detection of North American orthopoxviruses by real time-PCR. Virol J 8: 313.

12. Hazelton, PR, Gelderblom, HR.: 2003, Electron microscopy for rapid diagnosis of infectious agents in emergent situations. Emerg Infect Dis: 9: 294–303.

13. Hemmer CJ, Littmann M, Löbermann M, et al.: 2010. Human cowpox virus infection acquired from a circus elephant in Germany. Int J Inf Dis 14: Suppl 3.

14. Hosamani M, Scagliarini A, Bhanuprakash V. et al.: 2009, Orf: an update on current research and future perspectives. Exp Rev Anti-infect Ther 7: 879-93.

15. Inoshima Y, Morooka A, Sentsui H: 2000, Detection and diagnosis of parapoxvirus by the polymerase chain reaction. J Virol Meth 84: 201–208.

16. Kalthoff D, Bock WI, Hühn F, et al.: 2014, Fatal cowpox virus infection in cotton-top tamarins (Saguinus oedipus) in Germany. Vector Borne Zoonot 14: 303-305.

17. Kurth A, Straube M, Kuczka A. et al.: 2009, Cowpox Virus Outbreak in Banded Mongooses (Mungos mungo) and Jaguarundis (Herpailurus yagouaroundi) with a Time-Delayed Infection to Humans. PLoS ONE 4(9): e6883.

18. Lederman ER, Austin C, Trevino I, et al.: 2007, ORF virus infection in children: clinical characteristics, transmission, diagnostic methods, and future therapeutics. Pediat Inf Dis J 26: 740-4.

19. Rector A, Bossart GD, Ghim SJ, et al.: 2004, Characterization of a novel close-to-root papillomavirus from a Florida manatee by using multiply primed rolling-circle amplification: Trichechus manatus latirostris papillomavirus type 1. J Virol 78: 12698-702.

20. Rector A, Van Ranst M: 2013. Animal papillomaviruses. Virol 445: 213-223.

21. Saitou N, Nei M: 1987, The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.

22. Tamura K: 1992, Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. Mol Biol Evol 9: 678-687.

23. Tamura K, Stecher G, Peterson D, et al.: 2013, MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30: 2725-2729.

24. Tryland M, Okeke MI, Af Segerstad CH, et al.: 2011, Orthopoxvirus DNA in Eurasian lynx, Sweden. Emerg Inf Dis 17(4): 626-632.

25. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JA: 2007, Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 35(Web Server issue):W71-4.

26. von Bomhard W, Mauldin EA, Breuer W, et al.: 2011, Localized cowpox infection in a 5- month-old Rottweiler. Vet Dermatol 22: 11- 14.

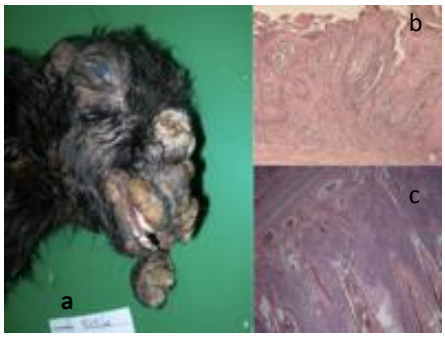

Figure 1. a) Multiple tumour-like lesions in a goat kid (9605-425) simultaneously infected with zoonotic poxviruses; b) hyperkeratosis, hyperplasia, acanthosis and ballooning degeneration of the epidermis (Hematoxylin-eosin. Obj. 50×); c) vascular proliferation and hypertrophic endothelia in the superficial dermis (Hematoxylin-eosin. Obj. 100×).

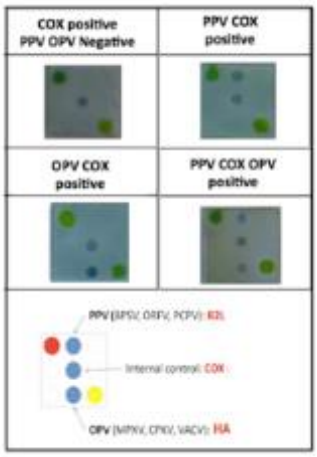

Figure 2. Mini-array results allowing the simultaneous detection of orthopoxviruses and parapoxviruses.

Table 1: Epiteliothropic viruses detected in thepathological lesions of diseased animals. The sequences of Orthopox detected in cows and goats were submitted to GENBANK and their accession numbers are indicated. * DPV = Deltapapillomavirus; XPV = Xipapillomavirus; EPV = Epsilonpapillomavirus; BPSV = Bovine papular stomatitis virus; PCPV = Pseudocowpox virus; ORFV = Orf virus; – = negative result.

Source/Animal ID	Papillomavirus	Parapoxvirus	Orthopoxvirus
Bovine			
25084-161	δ	–	–
31585-156	δ- ξ	–	–
75176-153	δ	–	–
85704-162	δ	–	–
10549-174	ξ	–	–
28580-287	δ- ξ	BPSV	–
6231-416	δ- ξ- ε	PCPV	–
14647-429	δ- ξ	BPSV	KM881110
33225-498	δ- ξ	PCPV	KM881111
50627-534	δ- ξ	BPSV	–
1818	ξ	PCPV	–
12748-610	δ	PCPV	–
Sheep			
70452-158	–	ORFV	–
70461-157	–	ORFV	–
73824-159	–	ORFV	–
73830-160	–	ORFV	–
13173-164	–	ORFV	–
13175-305	–	ORFV	–
13176-306	–	ORFV	–
79157-387	–	ORFV	–
Goat			
54836-349	–	ORFV	–
9605-425	–	ORFV	KM881108
32063-501	–	ORFV	KM881109
60945-544	–	ORFV	–

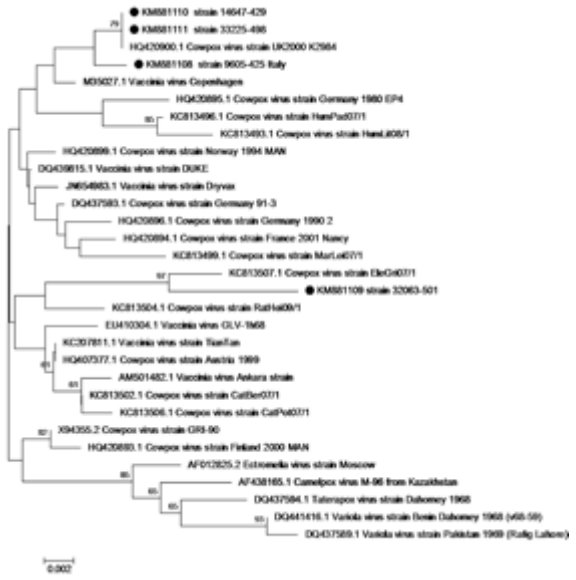

Figure 3: Phylogenetic tree generated from alignment of complete orthopoxvirus (OPV) Rpo 18 gene sequence using the neighbour-joining method and the Tamura 3-parameter model of nucleotide substitution, implemented in Mega 6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches (cutoff 60%). Dots indicate the strains object of the study.

Paper IV

Cervus elaphus papillomavirus (CePV1): new insights on viral evolution in deer

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Abstract

We identified a novel papillomavirus (CePV1) in a fibropapilloma of a 1,5 year old male red deer (*Cervus elaphus)* shot in the Italian Alps in Brescia province. PV particles were first observed by electron microscopy and PV DNA was then identified by PCR using degenerate primers. Subsequently we cloned the entire genome and determined its complete sequence. CePV1 genome is 8009 bp long and contains all 9 ORFs and the long untranslated regulatory region characteristic for Delta-papillomaviruses. Pairwise nucleotide alignments and phylogenetic analyses based on concatenated E1-E2-L1 ORFs allowed to determine the highest similarity with the *Capreolus caprelus* papillomavirus CcaPV1. The analysis of the host-parasite phylogenetic tree interactions suggest the co-divergence of CePV1 and *Cervus elaphus* while the identified topological incongruences leading us to speculate that CcaPV1 could eventually be the result of an earlier host switch event.

Keywords: Papillomavirus; Red deer, Cervus elaphus, evolution, genome

Introduction

Papillomaviruses (PVs) are small, non-enveloped, double stranded DNA viruses with circular genome that ranges between 6800 and 8400 bp. Eight to ten open reading frames (ORFs) are encoded, and transcribed from the same DNA strand with the same orientation. Translated proteins are classified as early (E) and late (L), based on their temporal expression (Gottschling, 2007). According to Bernard and co-workers (2010), the family Papillomaviridae contains 29 genera formed by 189 papillomavirus (PV) types isolated from humans (120 types), non-human mammals, birds and reptiles (64, 3 and 2 types, respectively) designated by Greek letters and classified based on genomic DNA homologies, especially of the late L1 structural protein (De Villiers et al., 2004; Bernard et al., 2010). The current classification of papillomaviruses is based on the comparison of complete L1 ORF nucleotide sequences, distinct Papillomavirus genera sharing less than 60% L1 ORF nucleotide sequence identity, while papillomavirus "species" within a genus approximately 60-70% (Bernard et al., 2010). Since 2010 the number of animal papillomaviruses has been gradually increasing, and new types have been detected, especially in mammals (Alberti et al., 2010; Hatama et al., 2011; Wood et al., 2011). Papillomaviruses constitute a diverse epitheliotropic group of viruses infecting the stratified squamous epithelia of skin and mucosa, and have been associated with warts and different types of cancer. PV genomes have been detected in papillomas of wild cervid species in Europe and North America (Shope et al., 1958; Lancaster and Sundberg, 1982; Moreno-Lopez et al., 1981; McDiarmid, 1975; Moar and Jarrett, 1985; Erdélyi et al., 2008; 2009a). The genomes of several cervid papillomaviruses have been already sequenced, such as the Deer (White-tailed deer - *Odocoileus virginianus*) Papillomavirus (Groff and Lancaster 1985), the Reindeer (*Rangifer tarandus*) Papillomavirus (Moreno-Lopéz et al., 1981), the European Elk (*Alces alces*) Papillomavirus (Stenlund et al., 1983) and the Western roe deer (*Capreolus capreolus*) papillomavirus (Erdélyi et al., 2008).

The majority of cervid papillomaviruses belong to the Delta genus, which includes also 4 Bovine papillomavirus 1 and 2 (BPV1 and BPV2) and Ovine papillomavirus 1 and 2 (OaPV1 and OaPV2) (Bernard et al., 2010). A common feature of these viruses is that they induce the development of skin tumours with a marked connective tissue component. Our study describes the complete genomic sequence of a papillomavirus identified in a cutaneous lesion of a 1,5 year old male red deer shot in the Italian Alps in Brescia province. This novel virus was named CePV1 following the nomenclature guidelines (Sundberg et al., 1996) for non-human 76 papillomaviruses.

Materials and methods Samples During an investigation on parapoxviruses causing diseases in wild ruminants of the central Italian Alps in Sondrio and Brescia provinces, the presence of a firm cutaneous tumour around 5.5 cm in size with a rough hairless pigmented surface was detected on the hind leg during post mortem inspection of a hunted 1,5 year old male red deer (Fig. 1). Tissue samples were excised from the tumour for diagnostic purposes and a first set of tissue samples was submitted to Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna (Brescia, Italy) for identification of the causative agent of the disease by negative staining (2,5% NaPT) transmission electron microscopy (TEM). In addition, 5-10 mm3 portions of the tumour were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were cut at 4μm and stained with haematoxillin eosin (HE) in order to characterise the lesion histologically. A further set of tissue samples was kept at -80°C for nucleic acid extraction.

Nucleic acid extraction and PCR DNA was extracted from 25 mg of tumour using NucleoSpin Tissue kit (Macherey and Nagel, Düren, Germany) following the manufacturer's instructions. Initially, a PCR reaction was performed to identify a fragment of approximately 480 bp of L1 gene of papillomaviruses using 5 primers FAP59/FAP64 as described by Forslund et al., 1999. Additionally, a PCR reaction using specific primers designed for

amplification of a 499 (BPV-1) or 497 bp (BPV-2) fragment spanning the E5 open reading frame (ORF) was carried out as described by Brandt et al., 2008. PCR products were purified using High pure PCR product kit (Roche, Milano, Italy) according to the manufacturer's instructions and sequenced directly in both directions with the corresponding primers.

Multiply primed rolling circle amplification Rolling Circle Amplification (RCA) combined with restriction enzyme analyses were carried out using the Templiphi amplification kit (GE Healthcare, Milano, Italy) following the protocols previously described by Rector et al., 2004 and Van Doorslaer et al., 2006, in order to identify papillomavirus genomes. Briefly, 5 µl of DNA extracted from the neoplastic tissue were mixed with 10 µl of sample buffer and subsequently heated for 3 minutes at 95°C, then transferred in an ice-bucket. Ten microliters of TempliPhi reaction buffer, 0.4 µ l of TempliPhi enzyme mix containing phi 29 DNA polymerase and random hexamers in 50% glycerol and 0,4 µl of 10mM dNTPs per sample were mixed and added to the cooled sample and the reaction was subsequently incubated for 16 hours at 30°C. The phi 29 polymerase was then inactivated at 65°C for 10 minutes. A digestion with restriction enzymes EcoRI, BamHI and SacI was subsequently performed and the products were run on a 0.8% agarose gel to visualise the presence of a DNA band consistent with the length of a papillomaviral genome or multiple bands with sizes adding up to this length.

DNA cloning and sequence analyses The 8000 bp DNA fragment obtained after the digestion of the RCA product with BamHI was cloned into pUC19 vector (Life Technologies, Monza, Italy) to generate pUC19/CePV1 IT1127. Briefly, 10 µl of the RCA product was digested with 100 units of BamHI for 3 hours and run on a 0.8% agarose gel, after which the fragment was extracted using the QIAquick Gel Extraction Kit (Qiagen, Jesi, Italy). The fragment was then ligated into the pUC19 vector that was previously cut with BamHI and dephosphorylated, using the Rapid DNA Dephos & Ligation Kit (Roche). The ligation

product was used to transform One Shot MAX Efficiency DH5Alpha™-T1R competent cells (Life Technologies). Bacteria were incubated for blue-white colony screening on agar plates containing X-gal, and white colonies were checked by BamHI digestion of miniprep DNA. One clone containing the 8 kb DNA fragment was selected. The complete genome of the *Cervus elaphus* papillomavirus (CePV1) was determined by primer-walking sequencing of the cloned DNA fragment (supplementary table S2, Supplementary Material), starting from the universal primer sites in the multiple cloning site of pUC19. In addition, in order to avoid any carry-over of possible point mutations generated during the amplification procedure, the original RCA amplicon was subjected to primer walking and sequencing using the same set of primers. Sequencing was performed on an ABI Prism 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Electropherograms were inspected and edited with 4Peaks 1.7.1, and contigs were prepared using SeqMan II (DNASTAR, Madison, WI, USA). Prediction of open reading frames (ORFs) was performed using the ORF Finder tool on the NCBI server of the National Institutes of Health (http://www.ncbi.nlm.nih.gov/gorf.html). Transcription factor binding sites and motifs were searched either manually or online using ScanProsite (http://www.expasy.ch/prosite). The molecular weight of the putative proteins was calculated using the ExPASy (Expert Protein Analysis System) Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). Pairwise sequence alignments and sequences similarities were calculated using the ClustalW (Thompson et al., 1994).

Phylogenetic analyses Nucleotide sequence alignments of the CePV1 ORFs with 89 other PVs were produced using ClustalW in DAMBE software version 5.0.29 (Xia and Xie, 2001). The nucleotide sequences were 7 first translated and aligned at the amino acid level to which alignment we fitted the original nucleotide sequences thus obtaining the final nucleotide sequence alignment (Larkin et al., 2007). Alternative alignments were also generated by omitting the third codon positions. This was done separately for the different ORFs, and the

unambiguously alignable parts of the E1, E2, L2, and L1 ORFs were pasted together in one compiled alignment. Genetic distances among the operational taxonomic units (OTUs) were computed as percent of total nucleotide differences or by the Kimura 2-parameters method using MEGA version 2.1 (Kumar et al., 2001), and were used to construct neighbour-joining (NJ) trees (Saitou and Nei, 1987). Statistical support for internal branches of the trees was evaluated by bootstrapping (Felsenstein, 1985). Maximum parsimony (MP) and Maximum likelihood (ML) trees were generated using the same software. Trees were edited using NJplot (Perriere and Gouy, 1996) and Treeview v. 1.5.2 (Page, 1996). Co-phylogenetic association of Delta-papillomaviruses and their hosts was assessed using the TreeMap program (http://taxonomy.zoology.gla.ac.uk/rod/treemap.html) which explores how the parasite tree fits onto the host tree by mixing four types of events: co-divergence, host switching, duplication or intra-host divergence of the parasite and sorting or extinction of the parasite lineage (Gottschling et al., 2011). With this aim we built the phylogenetic trees of viruses and their hosts generated using the distance matrix implemented in the software PAUP* version 4.0b10. The Delta papillomavirus tree was based on the concatenated alignment of E1, E2, L1 ORFs (supplementary table S1, Supplementary Material) while the host tree was generated after the alignment of the complete mitochondrial genomes (supplementary table S3, Supplementary Material).

Results Viral particles, identified by morphologic features as PV, were observed by negative stain electron microscopy in the material collected for pathologic examination from red deer IT 1127. Cross sections of the tumour mass were shiny and firm and histological examination classified the tumour as a fibropapilloma, showing hyperkeratosis and acanthosis of the epidermis, proliferation of fibroblasts and connective tissue. PCR reaction performed with FAP59 and FAP64 on DNA extracted from tissue sample IT 1127 confirmed the presence of PV DNA. Sequencing of the partial L1 PCR product obtained with FAP primers and corresponding to a portion of the L1 region allowed to establish the presence of a CcaPV1

variant, according to the criteria of De Villiers et al. (2004), with nucleotide and amino acid sequence identity of 91% and 96%, respectively. This new papillomavirus variant was designated CePV1. PCR performed with E5 primers detected the simultaneous presence of BPV DNA and direct sequencing of the amplified product demonstrated a co-infection with BPV-1. The full genome of CePV1 was obtained from sample IT 1127 by RCA that successfully generated linear double stranded tandem repeated copies of the circular DNA template. The digestion of the obtained double stranded DNA with a panel of restriction enzymes generated a pattern consistent with papillomavirus DNA: in particular BamHI, EcoRI and Sac I produced a single fragment of about 8 Kb. The 8 kb BamHI fragment was successfully cloned into pUC19 vector and the complete genome sequence was obtained by primer walking performed on the cloned DNA. The accuracy of the result was subsequently confirmed by direct sequencing of the original RCA amplicon. The nucleotide sequence of CePV1 full genome was deposited in GenBank using the National Centre for Biotechnology Information (NCBI, Bethesda, MD) BankIt v3.0 submission tool (http://www3.ncbi.nlm.nih.gov/BankIt/) under accession number JQ744282. All nine Delta papillomavirus ORFs have been detected in the CePV1 genome and after nucleotide and amino-acid alignments it was possible to define its similarity with other Delta papillomaviruses (Table 1). Overall, the highest nucleotide (nt) and amino acid (aa) identity was found with CcaPV1 and with reference to the single ORFs the highest similarity was found in E4 with 99.1% and 98.2% identity at nt and aa levels, respectively, while the lowest similarity was detected in E9 with 89.7% identity at both nucleotide and amino acid level. Similarly to CcaPV1, the GC content in the CePV1 genome is approximately 47%, while its length was found to be 8009 bp compared to the 8032 bp of CcaPV1. This different length is due to the deletion of 14 nucleotides in the untranslated LCR 200 region and of 9 nucleotides in L2 gene causing the lack of 3 amino acids (Ser, Ala, Arg) at positions 201 427, 428 and 479 of the encoded protein. The molecular masses of the putative proteins showed to 202 be

similar to those of CcaPV1. The predicted amino acid and nucleotide features are summarized in 203 table 2, the main differences found between CePV1 and CcaPV1 consisted in an additional E2 204 binding site (ACC-N5-7-GGT) and a further polyadenilation sites (AATAAA) detected in the CePV1 genome, respectively at positions 4424-4436 in L2 ORF and 1251-1256 in E1 ORF. Furthermore, TATA boxes have been identified in LCR and at nucleotide 60 (TATAAA) of E6 ORF as it was found in CcaPV1 and OaPV3 genomes and an additional box located in E1 ORF starting at position 1530. The E1 binding site (A (A/T)GATTGTTGTTAACAAT) shows a T at position two as it has been found in reindeer PV and OaPV2 while CcaPV1 has an A as in BPV-5. Finally, two additional Nuclear factor 1 (NF1) binding sites (CGGAA) have been identified at positions 490 in E6 ORF and 5658 in L2 ORF compared to CcaPV1 and an additional AP-1 (Activator Protein-1) binding sites (TGANTCA) was detected at nt 1682 (ORF E1). The pRB-binding domain (L-X-C-X-E) was absent in the putative E7 protein as in all Delta-papillomaviruses (Narechania et al., 2004) and the putative E4 protein showed the typical high proline content 215 (12.3%). Phylogenetic trees were obtained from a concatenated E1/E2/L1 nucleotide sequence alignment of CePV1 and 89 PV-types representative of the different PV genera and species (Figure 2). Nucleotide sequence alignments, based on the corresponding amino acid alignments, were constructed separately for the different ORFs. Regions where an unambiguous alignment could be obtained were included in one combined alignment of 2814 nucleotides (supplementary Table S1, Supplementary Material). The resulting neighbour-joining and maximum likelihood phylogenetic trees clustered the PVs in their respective genera, according to the new classification of PV (Bernard et al., 2010). In the trees, CePV1 appears as a novel Delta papillomavirus, closely related to CcaPV1 (*Capreolus capreolus papillomavirus* type 1), and therefore a representative of the 10 *Delta-5* species (Bernard et al., 2010). Maximum parsimony and Maximum Likelyhood trees constructed using the same nucleotide alignment were in agreement with neighbour-joining trees (data not shown). The comparison of host and

virus phylogenetic trees in TreeMap (Figure 3a) resulted in a tanglegram showing several topological incongruences while reconstruction (Figure 3b) identified two duplication events at the base of the host - virus tree, three co-speciation events and evidence of host switch events that shaped PV diversification in deer hosts.

Discussion

Papillomaviruses have been already identified in skin tumours of European elk, reindeer, roe deer, red deer and North American deer species (e.g. White-tailed deer). A common feature of these viruses is that they induce the development of fibropapillomas, skin tumours with a marked connective tissue component where fibroblasts appear to be the primary target cells of the virus. This is the first reported case of Papillomavirus infection in red deer in Italy, a disease that has been sporadically reported in other European Countries such as Spain, France, England, Austria and Hungary (Erdélyi et al., 2009a). The age of the affected animal and the pathological lesions were similar to those previously reported (Moar and Jarret 1985; Erdélyi 2009a) with no evidence of tumour metastasis. Our results identified a papillomavirus co-infection, as nucleid acid amplification evidenced the presence of both BPV-1 and CePV1 DNA. Nevertheless, quantitative PCR analysis indicated that CePV1 DNA yield was four logarithms higher than BPV-1 DNA (data not shown) and confirmed that the clinical lesions were ascribable to a productive CePV1 infection. The co-infection of a red deer with BPV-1 is not surprising, according to the already observed cross species transmission ability of this Delta papillomavirus (Nasir and Campo, 2008). The genetic organization of the CePV1 exhibits a typical Delta papillomavirus architecture with homologous genetic structures containing 9 ORFs encoding the regulatory early proteins and late structural proteins as well as the non-coding regulatory region (LCR). Phylogenetic analyses 11 allowed us to confirm that the red deer PV clusters within the Delta papillomavirus group and represent an additional PV variant closely related to CcaPV-1. Papillomaviruses are highly host specific,

and it has been generally assumed that these pathogens co-diverged with their mammal hosts so that related PVs infect related hosts. Delta papillomaviruses infecting ruminants are often quoted as an example for this statement; known as 256 Clay's rule but the absence of congruence between virus and host phylogenies has been suggested recently (Bravo *et al*. 2010; Gottshling et al., 2011; Mengual-Chuliá *et al.* 2012). In this study we also identified several topological incongruences between the phylogenetic trees of the mammal hosts and their viruses, and we suggest that CePV1 could have evolved during co-speciacion with its host *Cervus elaphus*. CePV1 is the closest relative of CcaPV1, identified in roe deer (*Capreolus capreolus*). According to Gilbert et al (2006) red deer and roe deer belong to 2 different subfamilies within the *Cervidae* family: the *Cervinae* and the *Capreolinae* and thus to the clades of Plesiometacarpalia and Telemetacarpalia. A similar incongruence was already reported for AaPV1 isolated from European elk (*Alces alces*) that showed to be the closest relative of reindeer PV (RPV) isolated from Rangifer tarandus, and not of OvPV1 isolated from *Odocoileus virginianus* as it would have been expected if host phylogenesis was congruent (Gottschling et al., 2011). Co-divergence plays an important evolutionary role in PVs and our data lead us to speculate that CePV1 can be the result of a co-speciation between the virus and its host. Co-speciation is not the only event that shaped PV diversification (Gottshling et al. 2011) in fact other events such as duplication and host switching can disrupt the topological congruence between the phylogenetic trees of Delta papillomaviruses and their hosts. The high similarity found between CePV1 and CcaPV1, seems to indicate that these PVs belong to the same ancestral lineage and our data provide evidence that CcaPV1 can be the result of a host switch event. Interspecies transmission of viruses is a broadly accepted phenomenon but it is still debated whether it requires adaptation to a new host during the early stages of infection or whether transmission itself could be a largely random process (Dennehy et al., 2006). The primary transmission route of cutaneous papillomaviruses is through 12 direct contact with contaminated environment or infected animals shedding the

virus from the surface of tumours. In European countries, where red deer fibropapillomas have been reported, there is no evidence of a similar infection in roe deer. At present Hungary is the only European country in which there is a coexistence of CePV1 and CcaPV1 infection in local deer population. Erdélyi and collaborators (2009b) speculated that the endemic nature of roe deer fibropapillomatosis in Hungary could be linked to the genetic differentiation pattern of European roe deer populations. An alternative explanation could be linked to peculiarities of deer habitat use and geography. The habitat use of deer has been widely studied in the European countries where the diseases has been detected (Birò et al., 2006; San José et al., 1997; Prokesòva et al., 2006), leading to demonstrate that inter-specific spatial interference between red and roe deer may vary on a wide scale also providing opportunities for virus transmission from one species to the other. Although habitat change due to agriculture and modern land use practices greatly modify the ecology of modern deer, with a potential to intensify inter-specific spatial interference, we must not forget that the current PV phylogeny reflects much older evolutionary processes. The mechanism of inter species transmission gaining a new hosts for CePV1 should be studied further in the light of the fact that other Delta papillomaviruses such as BPV-1 and 2 have already shown to be transmissible even to phylogenetically distant animal species. In conclusion, our results are consistent with the findings of Bravo and co-workers (2010), suggesting that the current PV classification system should be revised and an alternative naming approach should be considered, since host specificity is not always strictly maintained in animal papillomaviruses. Consequently, it is not correct to assume that the host species in which a PV was first detected is the original host. More sequence data from a larger number of deer viruses and extensive epidemiological studies will be required to better understand the evolution of deer 301 papillomaviruses and their hosts.

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REFERENCES

1) Alberti, A., Pirino, S., Pintore, F., Addis, M. F., Chessa, B., Cacciotto, C., Cubeddu, T., Anfossi, A., Benenati, G., Coradduzza, E., Lecis, R., Antuofermo, E., Carcangiu, L., Pittau. M., 2010. Ovis aries Papillomavirus 3: a prototype of a novel genus in the family Papillomaviridae associated with ovine squamous cell carcinoma. Virology. 407, 352-9.

2) Bernard, H.U., Burk, R.D., Chen, Z., Van Doorslaer, K., Hausen, H., De Villiers E.M., 2010. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendements. Virology. 401, 70-79.

3) Bíró, Z., Szemethy, L., Katona, K., Heltai, M., Peto, Z., 2006. Seasonal distribution of red deer (*Cervus elaphus*) in a forest-agriculture habitat in Hungary. Mammalia. 70–75.

4) Brandt, S., Haralambus, R., Schoster, A., Kirnbauer, R. e Stanek, C., 2008. Peripheral blood mononuclear cells represent a reservoir of bovine papillomavirus DNA in sarcoid-affected equines. J. Gen. Virol. 89, 1390-139.

5) Bravo, I.G, de Sanjosé, S., Gottschling, M., 2010. The clinical importance of understanding the evolution of papillomaviruses. Trends Microbiol. 18, 432-438.

6) Dennehy, J.J., Friedenberg, NA., Holt, R.D., Turner, P.E., 2006. Viral ecology and the maintenance of novel host use. Am. Nat. 167, 429-439.

7) De Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U., Zur Hausen, H., 2004. Classification of papillomaviruses. Virology. 324, 17-27. 327

8) Erdélyi, K., Bálint, Á., Dencső, L., Dán, Á., Ursu., K. 2008. Characterisation of the first complete genome sequence of the roe deer (Capreolus capreolus) papillomavirus. Virus Res. 135, 307-311.

9) Erdélyi, K., Gál, J., Sugár, L., Ursu, K., Forgách, P., Szeredi, L., Steineck, T. 2009a. Papillomavirus associated fibropapillomas of red deer (Cervus elaphus). Acta Vet. Hung. 57, 332 337–344. 15

10) Erdélyi, K., Dencső, L., Lehoczki, R., Heltai, M., Sonkoly, K., Csányi, S., Solymosi, N., 2009b. Endemic papillomavirus infection of roe deer (Capreolus capreolus). Vet. Microbiol. 138, 20- 26.

11) Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evol. 39, 783-791.

12) Forslund, O., Antonsson, A., Nordin, P., Stenquist, B., Hansson, B.G., 1999. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J. Gen. Virol. 80, 2437-43.

13) Gilbert, C., Ropiquet, A., Hassanin, A., 2006. Mitochondrial and nuclear phylogenies of Cervidae (Mammalia, Ruminantia): Systematics, morphology, and biogeography. Mol. Phylogenet. Evol. 40, 101-17.

14) Gottschling, M., Stamatakis, A., Nindl, I., Stockfleth, E., Alonso, A., Bravo, I.G. 2007.

Multiple Evolutionary Mechanisms Drive Papillomavirus Diversification. Mol. Biol. Evol. 24, 1242-1258.

15) Gottschling, M., Göker, M., Stamatakis, A., Bininda-Emonds, O.R., Nindl, I., Bravo, I.G., 2011. Quantifying the phylodynamic forces driving papillomavirus evolution. Mol. Biol. Evol. 8, 2101-13.

16) Groff, D.E., Lancaster, W.D., 1985. Molecular cloning and nucleotide sequence of deer papillomavirus. J. Virol. 56, 85-91.

17) Hatama, S., Ishihara, R., Ueda, Y., Kanno, T., Uchida, I., 2011. Detection of a novel bovine papillomavirus type 11 (BPV-11) using xipapillomavirus consensus polymerase chain reaction primers. Arch. Virol. 156, 1281-1285.

18) Hall, T.H., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser (Oxf). 41, 95-98.

19) Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17, 1244-1245. 16

20) Lancaster, W.D., Sundberg, J.P., 1982. Characterization of papillomaviruses isolated from cutaneous fibromas of white-tailed deer and mule deer. Virology. 123, 212-216.

21) Larkin, MA., Blackshields, G., Brown, NP., Chenna, R., McGettigan, PA., McWilliam, H., Valentin, F., Wallace, IM., Wilm, A., Lopez, R., Thompson, JD., Gibson, TJ., Higgins, DG., 2007. Clustal W and Clustal X version 2.0. Bioinformatics. 23(21), 2947-8.

22) Mengual-Chuliá, B., García-Pérez, R., Gottschling, M., Nindl, I., Bravo, I.G., 2012. Novel animal papillomavirus sequences and accurate phylogenetic placement. Mol. Phylogenet. Evol. 65, 883-91.

23) Moar, M. H., Jarrett, W. F., 1985. A cutaneous fibropapilloma from a red deer (Cervus elaphus) associated with a papillomavirus. Intervirol. 24, 108-118.

24) Moreno-Lopez, J., Pettersson, U., Dinter, Z., Philipson, L., 1981. Characterization of a papilloma virus from the European elk (EEPV). Virology. 112, 589-95.

25) McDiarmid, A., 1975. Some disorders of wild deer in the United Kingdom. Vet. Rec. 97, 6-9.

26) Narechania, A., Terai, M., Chen, Z., DeSalle, R., Burk, R.D., 2004. Lack of the canonical pRB-binding domain in the E7 ORF of artiodactyl papillomaviruses is associated with the 374 development of fibropapillomas. J. Gen. Virol. 85, 1243-1250.

27) Nasir, L., Campo, M.S., 2008. Bovine papillomaviruses: their role in the aetiology of cutaneous tumors of bovids and equids. Vet. Dermatol. 19, 243-254.

28) Page, R.D.M., Clayton, D.H., Paterson, A.M., 1996. Lice and cospeciation: a response to Barker. Int. J. Parasitol. 26, 213-218.Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12 (4), 357–358.

29) Perriere, G., Gouy, M., 1996. WWW-query: an on-line retrieval system for biological sequence banks. Biochimie. 78, 364-369.

30) Prokešová, I., Barančeková, M., Homolka, M., 2006. Density of red and roe deer and their distribution in relation to different habitat characteristics in a floodplain forest. Folia Zool. 55, 1–14. 17

31) Rector, A., Bossart, G.D., Ghim, S.J., Sundberg, J.P, Jenson, A.B., Van Ranst, M., 2004. Characterization of a novel close-to-root papillomavirus from a Florida manatee by using multiply primed rolling-circle amplification: Trichechus manatus latirostris papillomavirus type 1. J. Virol. 78, 12698-702.

32) San José, C., Braza, F., Aragón, S. & Delibes, J. R., 1997. Habitat use by roe and red deer in Southern Spain. Misc. Zool. 20, 27-38.

33) Shope, R.E., Mangold, R., Macnamara, L.G., Dumbell, K.R., 1958. An infectious cutaneous fibroma of the Virginia whitetailed deer (Odocoileus virginianus). J. Exp. Med. 108, 797-802.

34) Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

35) Stenlund, A., Moreno-Lopez, J., Ahola, H., Pettersson, U., 1983. European elk papillomavirus: characterization of the genome, induction of tumors in animals, and transformation in vitro. J Virol. 48, 370-376.

36) Sundberg, J. P., Van Ranst, M., Burk, R. D., Jenson, A. B., 1996. The nonhuman (animal) papillomaviruses: host range, epitope conservation, and molecular diversity. In: von Krogh G. & Gross. G. Boca Raton, FL: CRC Press (Eds.). Human Papillomavirus Infections. Dermatol. Venereol. 47-68.

37) Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

38) Van Doorslaer, K., Rector, A., Vos, P., Van Ranst, M., 2006. Genetic characterization of the Capra hircus papillomavirus: a novel close-to-root artiodactyl papillomavirus. Virus Res. 118,164-169.

39) Wood, C.E., Tannehill-Gregg, S.H., Chen, Z., Doorslaer, K., Nelson, D.R., Cline, J.M, Burk, R.D., 2011. Novel betapapillomavirus associated with hand and foot papillomas in a cynomolgus macaque. Vet. Pathol. 48, 731-736. 18

40) Xia, X., Xie, Z., 2001. DAMBE: software package for data analysis in molecular biology and evolution. J. Hered. 92, 371-373.

Figure 1: Nodular hairless mass covered with dark and dry skin localised on the inner surface of a hind leg.

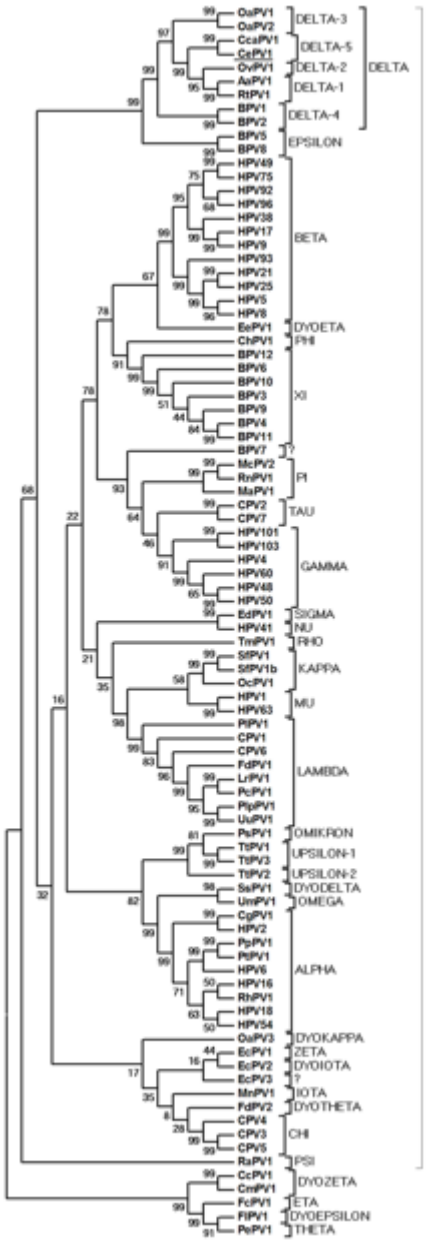

Figure 2: Coinciding NJ and ML trees based on a 2814 bp concatenated E1/E2/L1 nucleotide sequence alignment of CePV1 and other animal and human PV types. The numbers at the internal nodes represent the bootstrap support values, determined for 10,000 iterations with the neighbour-joining method.

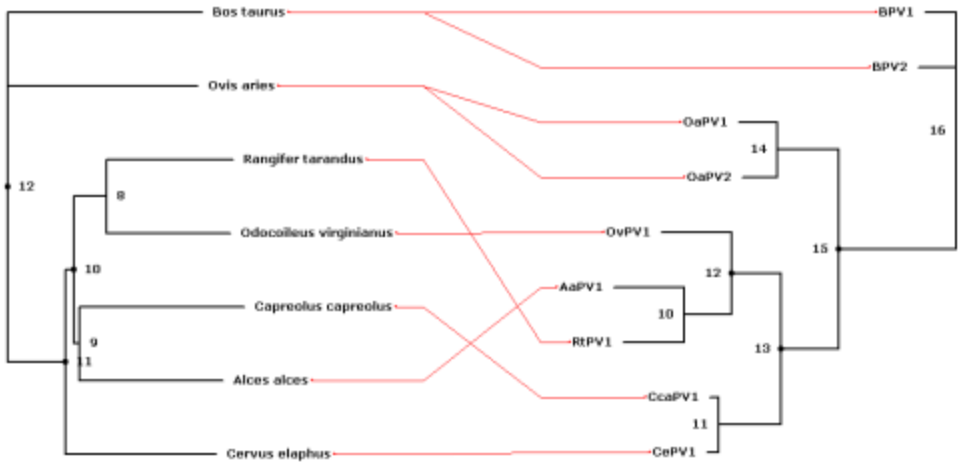

Figure 3: a) –upper-Congruence between Delta-papillomaviruses (right) and mammal host species (left). The Tanglegram built with TreeMap links the phylograms of PVs with their hosts, central linking lines represent specific virus-host associations. 3 b)-lower- Reconstruction showing co-divergence (black squares), duplication (black dots) and host switch events (black arrows).

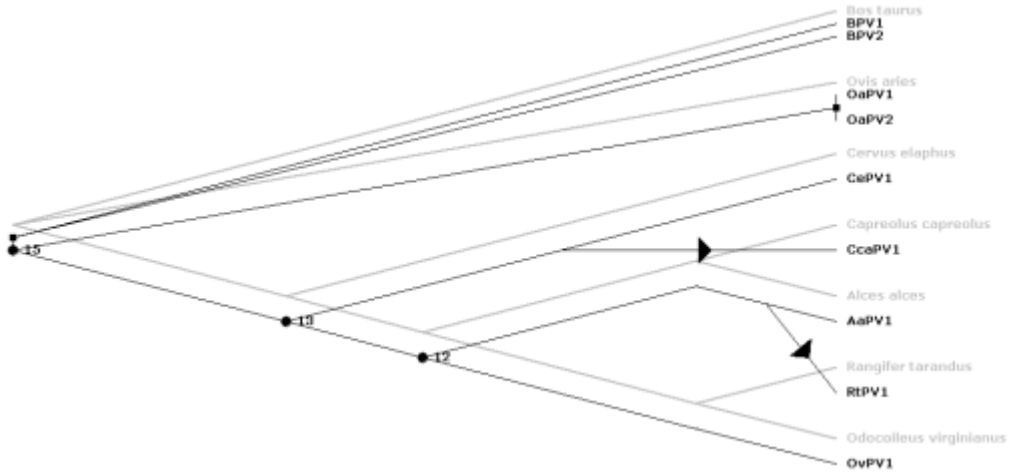

Table 1: Nucleotide and amino acid identity among Delta-papillomaviruses.

CePV1	CcPV1	OvPV	RtPV	AaPV	OaPV2	OaPV1	BPV1	BPV2
E6 (405)	96(92,6)	54,3(43.7)	56,9(51,9)	58,1(50,4)	50,3(41,1)	50,3(40,3)	56,6 (46,2)	55,6(46,9)
E7 (327)	96,3(95,4)	55,6(41,2)	41,7(11,9)	58,2(46,6)	54,2 (46)	54,3(44)	56(46,8)	54,1(45)
E1 (1839)	96,5(96,2)	71,5(69,7)	70,8(68,6)	69,1(66,6)	68,9(65,5)	68,3(64,7)	64,1(59)	63,8(59,6)
E2 (1224)	97,6(98)	65,3(55,6)	63,5(54,6)	63,4(51,1)	60,4(55,9)	63,2(55)	59,9(49,2)	59,2 (34,6)
E4 (342)	99,1(98,2)	65,8(54,5)	63(52,3)	61,7(57,7)	71,2	69,1 (58)	57,8(32,7)	55,6(37,6)
E5 (141)	93,6(91,5)	59,7(37,8)	55,2(44,4)	59,8(43,2)	57,1(46,8)	53,9(46,8)	57,5 (37,8)	57,5 (37,8)
E9 (174)	89,7(89,7)	52 (31)	49,6 (31,6)	44,3 (34,9)	x	x	44,9 (17,6)	43,1(19,6)
L2 (1455)	92,9(93,2)	67,1 (61,1)	64,1(64,2)	67 (66,5)	61,1(54,6)	60,2 (56)	54,5(48,9)	57,2 (50,9)
L1 (1524)	94,3(97,2)	71,4(77,1)	71,9(80,2)	72,3(79,2)	69,3(74,7)	67,5(72,9)	68,6(75,4)	68,9(74,7)

Table 2: Nucleotide and amino acid features of CePV genome. Underlined positions represent characteristic motifs which are not present in CcaPV1.

Predicted nt features	**CcaPV1** **GenBank n. EF680235**	**CcePV1 (IT-1127)** **GenBank n. JQ744282**
genome size (bp)	8032	8009
GC content (%)	47,31	47
E2 binding site (ACC-N_{5-7}-GGT)	73 (LCR); 231 (E6); 3770 (E2); 4552 (L2); 7476 (LCR); 7610 (LCR); 7661 (LCR): 7711 (LCR); 7794 (LCR); 7818 (LCR); 7858 (LCR); 7982 (LCR)	103 (E6); 3642 (E2); 4424 (L2); 4905 (L2); 7336 (LCR); 7470 (LCR); 7521 (LCR); 7571 (LCR); 7654 (LCR); 7678 (LCR); 7707(LCR); 7831 (LCR); 7954 (LCR)
E1 binding site (A(A/T)GATTGTTGTTAACAAT)	AAGATTGTTGTTAACAAT	ATGATTGTTGTTAACAAT
Poly adenilation sites (AATAAA)	4351 (E9); 6399 (L1); 7704(LCR); 7429(LCR);	4223(E9); 6262(L1); 7292(L1); 7564(LCR); 1251(E1)
Tata box (TATAAA or TATA(A/T)A(A/T)	60 (LCR)	1530 (E1); 7941(LCR)
SP1 binding sites (GGCGGG)	1001 (E1)	872 (E1)
NF1 binding sites (CGGAA)	3542(E2); 3339(E2/4); 5081(L2);	490(E6); 3211(E2/4); 3414 (E2); 4397 (L2) ;4871(L2); 5658 (L2)
AP1 binding site (TGANTCA)	532 (E6/7)	403(E6/7); 1682 (E1)
Predicted aa-feature		
ATP-dependent helicase motifs in E1 (GPPNTGKS)	439-446 aa	413-563 aa
zinc-binding motifs in E6 ($CX_2CX_{29}CX_2C$)	11-47 aa; 83-119 aa	11-47 aa; 83-119 aa
zinc-binding motifs in E7 ($CX_2CX_{29}CX_2C$)	65-101 aa	65-101 aa
Leucine zipper domain in E1 (LX_{5-7} LX_{5-7} $LX_{5-7}L$)	259-280 aa 266-287 aa	259-280 aa 266-287 aa
Leucine zipper domain in E2 (LX_{5-7} LX_{5-7} $LX_{5-7}L$)	/	/
E4 proline content	11,4%	12,3%

Table S1: Scientific names, host species, lesion/isolation sites, phylogeny, GENBANK accession numbers, and references of the 89 papillomaviruses considered in the phylogenetic analyses. Papillomavirus nomenclature and phylogeny are indicated according to the new proposal for taxonomic amendments as in: "Bernard, H. U., R. D. Burk, Z. Chen, K. Van Doorsiaer, H, zur Hausen, and E. M. de Villers. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology: doi:10.1016/j.virol.2010.02.002".

Scientific name	**Scientific abbreviation**	**Host**	**Lesion/isolation site**	**Phylogeny**	**GenBank Accession Number**	**Reference (* direct submission)**
Alces alces papillomavirus 1	AaPV1	*Alces alces* (Linnaeus, 1758) (Cervidae, Artiodactyla)	Epithelial layer of cutaneous warts	Delta-1	M15953	Ahola *et al*. (1986)
Bos taurus Papillomavirus 1	BPV1	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Fibropapilloma of skin	Delta-4	X02346	Chen *et al*. (1982)
Bos taurus Papillomavirus 2	BPV2	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Fibropapilloma of skin	Delta-4	M20219	Groff and Lancaster (1986)*
Bos taurus Papillomavirus 3	BPV3	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Hyperplasic epithelial warts	Xi	AF486184	Terai *et al*. (2002)
Bos taurus Papillomavirus 4	BPV4	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Esophagus papilloma	Xi	X05817	Patel *et al*. (1987)
Bos taurus Papillomavirus 5	BPV5	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	''Rice grain'' papilloma of benign tumor of teat	Epsilon	AF457465	Terai *et al*. (2002)
Bos taurus Papillomavirus 6	BPV6	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Frond epithelial papillomas of udder	Xi	AJ620208	Jarrett *et al*. (1984)
Bos taurus Papillomavirus 7	BPV7	*Bos taurus* (Linnaeus, 1758)	Teat	?	DQ217793	Ogawa *et al*. (2004)

		(Bovidae, Artiodactyla)				
Bos taurus Papillomavirus 8	BPV8	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Papillomas	Epsilon	DQ098913	Tomita *et al*. (2007)
Bos taurus Papillomavirus 9	BPV9	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Epithelial squamous papilloma lesions on teats	Xi	AB331650	Hatama *et al.* (2008)
Bos taurus Papillomavirus 10	BPV10	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Epithelial squamous papilloma lesions on teats	Xi	AB331651	Hatama *et al.* (2008)
Bos taurus Papillomavirus 11	BPV11	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	cutaneous warts	Xi	AB543507	Hatama *et al.* (2011)
Bos taurus Papillomavirus 12	BPV12	*Bos taurus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Epithelial papilloma located on the tongue of an infected cow	Xi	JF834523	Zhu *et al*. (2012)
Canis familiaris oral Papillomavirus	CPV1	*Canis familiaris* (Linnaeus, 1758) (Canidae, Carnivora)	Papilloma of 5-month-old female beagle	Lambda-2	D55633	Isegawa *et al*. (1994)
Canis familiaris Papillomavirus 2	CPV2	*Canis familiaris* (Linnaeus, 1758) (Canidae, Carnivora)	Foot pad papilloma of a Golden retriever	Tau	AY722648	Yuan *et al*. (2007)
Canis familiaris Papillomavirus 3	CPV3	*Canis familiaris* (Linnaeus, 1758) (Canidae, Carnivora)	Skin lesions from 7-year-old Rhodesian ridgeback with canine EV and in situ squamous cell carcinoma	Chi-1	DQ295066	Tobler *et al.* (2006)
Canis familiaris Papillomavirus 4	CPV4	*Canis familiaris* (Linnaeus, 1758) (Canidae, Carnivora)	Biopsy specimen of pigmented lesions from European pug	Chi-2	EF584537	Tobler *et al.* (2008)*
Canis familiaris Papillomavirus 5	CPV5	*Canis familiaris* (Linnaeus, 1758) (Canidae, Carnivora)	Pigmented plaques	Chi-1	FJ492743	Lange et al. (2009a)
Canis familiaris Papillomavirus 6	CPV6	*Canis familiaris* (Linnaeus, 1758)	Inverted papillomas	Lambda-3	FJ492744	Lange et al. (2009a)

		(Canidae, Carnivora)				
Canis familiaris Papillomavirus 7	CPV7	*Canis familiaris* (Linnaeus, 1758) (Canidae, Carnivora)	Exophytic warts, *in situ* SCC	Tau	FJ492742	Lange et al. (2009a)
Capreolus capreolus Papillomavirus 1	CcaPV1	(*Capreolus capreolus*, Linnaeus 1758) (Odocoileinae, Cervidae, Ungulata)	Fibropapillomatous skin lesions	Delta-5	NC_011051	Erdélyi *et al.* (2008)
Capra hircus Papillomavirus 1	ChPV1	*Capra hircus* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Healthy skin of year-old female	Phi	DQ091200	Van Doorslaer *et al*. (2006)
Caretta caretta Papillomavirus 1	CcPV1	*Caretta caretta* (Linnaeus, 1758) (Cheloniidae, Testudines)	Epidermal lesions	Dyozeta	EU493092	Herbst *et al.* (2009)
Chelonia mydas Papillomavirus 1	CmPV1	*Chelonia mydas* (Linnaeus, 1758) (*Chelonidae, Testudines)*	Epidermal lesions	Dyozeta	EU493091	Herbst *et al.* (2009)
Colobus guereza papillomavirus 1	CgPV1	*Colobus guereza*, (Rüppell, 1835) (Cercopithecidae, Primates)	Hand and foot papillomas	Alpha-14	GU014532	Wood *et al.* (2011)
Equus caballus Papillomavirus 1	EcPV1	*Equus caballus* (Linnaeus, 1758) (Equidae, Perissodactyla)	Cutaneous papilloma	Zeta	AF498323	Ghim *et al*. (2004)
Equus caballus Papillomavirus 2	EcPV2	*Equus caballus* (Linnaeus, 1758) (Equidae, Perissodactyla)	Genital neoplasia	Dyoiota	Eu503122	Scase et al. (2010)
Erethizon dorsatum Papillomavirus 1	EdPV1	*Erethizon dorsatum* (Linnaeus, 1758) (Erethizontidae, Rodentia)	Epidermal hyperplasia, with acanthosis and orthokeratotic hyperkeratosis, from multiple white to light brown lobulated, raised, firm masses on foot pads	Sigma	AY684126	Rector *et al*. (2005a)
Erinaceous europaeus Papillomavirus 1	EePV1	*Erinaceus europaeus* (Linnaeus, 1758) (Erinaceidae, Insectivora)	free-ranging adult male facial hair follicles	Dyoeta	FJ379293	Schulz *et al.* (2009)

Felis domesticus Papillomavirus 1	FdPV1	*Felis silvestris* (Schreber, 1775) (Felidae, Carnivora)	hyperkeratotic cutaneous lesions of a Persian domestic cat	Lambda-1	AF480454	Tachezy *et al.*, (2002a)
Felis domesticus Papillomavirus 2	FdPV2	*Felis silvestris* (Schreber,1775) (Felidae, Carnivora)	Bowenoid in situ squamous cell carcinoma	Dyotheta	EU796884	Lange *et al*. (2009b)
Fringilla coelebs Papillomavirus	FcPV1	*Fringilla coelebs* (Linnaeus, 1758) (Fringillidae, Passerida)	Epithelial warts on tarsus and feet	Eta	AY057109	Terai *et al*. (2002)
Francolinus leucoscepus Papillomavirus 1	FlPV1	*Francolinus leucoscepus* (Grey, 1867) (Phasianidae, Galliformes)	Healthy skin	Dyoepsilon	Eu188799	Van Doorslaer *et al.* (2009)
Human Papillomavirus 1, HPV1	HPV1	*Homo sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Plantar wart	Mu-1	NC_001356	Danos *et al*. (1982)
Human Papillomavirus 101, HPV101	HPV101	*H. sapiens*(Linnaeus, 1758) (Hominidae, Primates)	Cervicovaginal cells from a 34-year-old woman with intraepithelial neoplasia grade 3	Gamma-6	DQ080081	Chen *et al.* (2007)
Human Papillomavirus 103, HPV103**	HPV103	*H. sapiens*(Linnaeus, 1758) (Hominidae, Primates)	Cervicovaginal cells from 30-year-old woman with normal cytology	Gamma-6	DQ080078	Chen *et al.* (2007)
Human Papillomavirus 16, HPV16	HPV16	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Invasive cervical carcinoma	Alpha-9	K02718	Seedorf *et al.* (1985)
Human Papillomavirus 17, HPV17**	HPV17	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Not specified	Beta	X74469	Delius and Hoffman (1994)
Human Papillomavirus 18, HPV18	HPV18	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Cervical cancer	Alpha-7	X05015	Cole and Danos (1987)
Human Papillomavirus 2, HPV2	HPV2	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Pooled DNA from warts of different patients	Alpha-4	X55964	Hirsch-Behnam *et al*. (1990)

Human Papillomavirus 21, HPV21**	HPV21	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Skin warts of an epidermodysplasia verruciformis (EV) patient	Beta	U31779	Kremsdorf *et al.* (1984)
Human Papillomavirus 25, HPV25**	HPV25	*H. sapiens*(Linnaeus, 1758) (Hominidae, Primates)	Not specified	Beta	X74471	Delius and Hoffman (1994)
Human Papillomavirus 38, HPV38**	HPV38	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Superficial spreading malignant melanoma of an immunosuppressed patient	Beta	U31787	Scheurlen *et al*. (1986)
Human Papillomavirus 4, HPV4	HPV4	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Wart from EV patient Not specified	Gamma-1	X70827	Egawa *et al*. (1993)
Human Papillomavirus 41, HPV41	HPV41	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Disseminated facial, perianal, and foot warts from 15-year-old girl, skin carcinomas, and premalignant keratoses	Nu-1	X56147	Hirt *et al*. (1991)
Human Papillomavirus 48, HPV48	HPV48	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Squamous cell carcinoma of the hand of immunosuppressed 36-year-old woman	Gamma-2	U31789	Muller *et al*. (1989)
Human Papillomavirus 49, HPV49	HPV49	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Pooled flat warts	Beta-3	X74480	Delius and Hofmann (1994)
Human Papillomavirus 5, HPV5	HPV5	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Flat wart from EV patient	Beta-1	M17463	Zachow *et al*. (1987)
Human Papillomavirus 50, HPV50	HPV50	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Actinic keratosis from EV patient	Gamma-3	U31790	Favre *et al*. (1989)
Human Papillomavirus 54, HPV54	HPV54	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Penile Buschke-Lo¨wenstein tumour coexisting with Condylomata acuminata	Alpha-13	U37488	Farmer (1990)*
Human Papillomavirus 6, HPV6	HPV6	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Condyloma acuminata	Alpha-10	X00203	Schwarz *et al*. (1983)

Human Papillomavirus 60, HPV60	HPV60	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Keratinous plantar cyst	Gamma-4	U31792	Matsukura *et al*. (1992)
Human Papillomavirus 63, HPV63	HPV63	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Pooled punctuate keratotic lesion	Mu-2	X70828	Egawa *et al*. (1993)
Human Papillomavirus 75, HPV75**	HPV75	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Not specified	Beta	Y15173	Delius *et al*. (1998)
Human Papillomavirus 8, HPV8**	HPV8	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Not specified	Beta	M12737	Fuchs *et al*. (1986)
Human Papillomavirus 9, HPV9	HPV9	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Flat wart from EV patient	Beta-2	X74464	Delius and Hofmann (1994)
Human Papillomavirus 92, HPV92	HPV92	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Basal cell carcinoma of 89-year-old man	Beta-4	AF531420	Forslund *et al*. (2003)
Human Papillomavirus 93, HPV93**	HPV93	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Solar keratosis on dorsum of hand of a non-immunosuppressed male	Beta	AY382778	Vasiljevic *et al*. (2007)
Human Papillomavirus 96, HPV96**	HPV96	*H. sapiens* (Linnaeus, 1758) (Hominidae, Primates)	Perilesional skin of a squamous cell carcinoma in situ,	Beta	AY382779	Vasiljevic *et al*. (2007)
Lynx rufus Papillomavirus 1	LrPV1	*Lynx rufus* (Schreber, 1777) (Felidae, Carnivora)	Tongue lesion	Lambda-1	AY904722	Rector *et al*. (2007)
Macaca mulata Papillomavirus 1, MmPV1	RhPV1	*Macaca mulata* (Zimmermann, 1780) (Cercopithecidae, Primates)	Penile squamous cell carcinoma	Alpha-12	M60184	Ostrow *et al*. (1991)
Mastomys coucha Papillomavirus 2	McPV2	*M. coucha* (Smith, 1834) (Muridae, Rodentia)	Anal lesion	Pi-1	DQ664501	Nafz *et al*. (2008)
Mastomys natalensis Papillomavirus 1	MnPV1	*Mastomys natalensis* (Smith, 1834)	Benign and malignant proliferations of adult animals	Iota	U01834	Tan *et al*. (1994)

		(Muridae, Rodentia)				
Mesocricetus auratus Papillomavirus 1	MaPV1	*Mesocricetus auratus* (Waterhouse, 1839) (Muridae, Rodentia)	Lesions in lingual mucosa	Pi-2	E15111	Iwasaki *et al*. (1997)
Odocoileus virginianus Papillomavirus 1	OvPV1	*Odocoileus virginianus* (Zimmermann, 1780) (Cervidae, Artiodactyla)	Pooled fibromas of females	Delta-2	M11910	Groff and Lancaster (1985)
Oryctolagus cuniculus Papillomavirus 1	OcPV1	*Oryctolagus cuniculus* (Linnaeus, 1758) (Leporidae, Lagomorpha)	Pooled from lesions at underside of tongue	Kappa-1	AF227240	Christensen *et al*. (2000)
Ovis aries Papillomavirus 1	OaPV1	*Ovis aries* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Fibropapilloma	Delta-3	U83594	Karlis *et al*. (2000)*
Ovis aries Papillomavirus 2	OaPV2	*Ovis aries* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Fibropapilloma	Delta-3	U83595	Karlis *et al*. (2000)*
Ovis aries Papillomavirus 3	OaPV3	*Ovis aries* (Linnaeus, 1758) (Bovidae, Artiodactyla)	Squamous cell carcinoma	Dyokappa		Alberti *et al.* (2010)
Pan paniscus Papillomavirus 1	PpPV1	*Pan paniscus* (Schwartz, 1929) (Hominidae, Primates)	Focal epithelial hyperplasia-like disease in oral cavity	Alpha-10	X62844	Van Ranst *et al*. (1991)
Pan troglodytes papillomavirus 1, PtPV1**	PtPV1	*Pan troglodytes* (Blumenbach, 1775) (Hominidae, Primates)	Focal epithelial hyperplasia-like disease	Alpha-10	AF020905	Scinicariello F, Soza I, Brasky KM and Hilliard JK (1997)*
Procyon lotor Papillomavirus 1	PlPV1	*Procyon lotor* (Linnaeus, 1758) (Procyonidae, Carnivora)	Papillomatous skin lesions of an adult raccoon	Lambda-4	AY763115	Rector *et al*. (2005b)
Puma concolor papillomavirus 1	PcPV1	*Puma concolor* (Linnaeus 1771) (Felidae, Carnivora)	Papillomatous lesion under the tongue	Lambda-1	AY904723	Rector *et al*. (2007)
Psittacus erithacus Papillomavirus 1	PePV1	*Psittacus erithacus timneh* (Fraser, 1844)	Cutaneous lesion at head	Theta	AF420235	Tachezy *et al*. (2002b)

		(Psittacidae, Psittaciformes)				
Panthera leo persica Papillomavirus 1	PlpPV1	*Panthera leo persica* (Meyer, 1826) (Felidae, Carnivora)	Papilloma lesions of tongue	Lambda-1	AY904724	Rector *et al*. (2007)
Phocaena spinipinnis Papillomavirus 1	PsPV1	*Phocaena spinipinnis* (Burmeister, 1865) (Phocoenidae, Cetacea)	Genital slit wart	Omikron	AJ238373	Van Bressem *et al.* (2007)
Rangifer tarandus papillomavirus 1,	RtPV1	*Rangifer tarandus* (Linnaeus, 1758) (Cervidae, Artiodactyla)	Epithelial layer of a cutaneous fibropapilloma	Delta-1	AF443292	Moreno-Lope´z *et al*. (1987);
Rousettus egyptiacus Papillomavirus 1	RaPV1	*Rousettus egyptiacus* (E. Geoffroy, 1810) (Pteropodidae, Chiroptera)	Basosquamous carcinoma on the left wing membranes	Psi	DQ366842	Rector *et al*. (2006)
Rattus norvegicus papillomavirus 1	RnPV1	*Rattus Norvegicus* (Berkenhout, 1769)	Oral mucosa	Pi-1	Gq180114	Schulz *et al*. (2009b)
Sus scrofa Papillomavirus 1	SsPV1	*Sus scrofa domestica* (Linnaeus, 1758) (Muridae, Rodentia)	Healthy skin of a female domestic pig	Dyodelta	EF395818	Stevens *et al*. (2008a)
Sylvilagus floridanus Papillomavirus 1,	SfPV1	*Sylvilagus floridanus* (J. A. Allen, 1890) (Leporidae, Lagomorpha)	Pooled papillomas	Kappa-2	K02708	Giri *et al*., 1985
Sylvilagus floridanus Papillomavirus 1,	SfPV1b**	*Sylvilagus floridanus* (J. A. Allen, 1890) (Leporidae, Lagomorpha)	Pooled papillomas	Kappa-2	AJ243287	Salmon *et al*. (2000)
Trichechus manatus latirostris Papillomavirus 1	TmPV1	*Trichechus manatus latirostris* (Harlan, 1824) (Trichechidae, Sirenia)	Sessile papillomatous skin lesion of female	FL, United States	AY609301	Rector *et al*. (2004)
Tursiops truncatus Papillomavirus 1, TtPV1	TtPV1	*Tursiops truncatus* (Montagu, 1821) (Delphinidae, Cetacea)	Genital wart on penis	Upsilon-1	EU240894	Rector *et al*. (2008)
Tursiops truncatus Papillomavirus 2, TtPV2	TtPV2	*Tursiops truncatus* (Montagu, 1821) (Delphinidae, Cetacea)	Genital Condylomata	Upsilon-2	AY956402	Rehtanz *et al*. (2006)
Tursiops truncatus Papillomavirus 3, TtPV3	TtPV3	*Tursiops truncatus* (Montagu, 1821) (Delphinidae, Cetacea)	Genital wart on penis	Upsilon-1	EU240895	Rector *et al*. (2008)

Uncia Uncia papillomavirus 1	UuPV1	*Uncia uncia* (Schreber 1775) (Felidae, Carnivora)	Lesion of the lower lip	Lambda-1	Dq180494	Rector *et al*. (2007)
Ursus maritimus Papillomavirus 1	UmPV1	*Ursus maritimus* (Phipps 1784) (Ursidae, Carnivora)	Oral mucosa of a polar bear	Omega	EF536349	Stevens *et al*. (2008b)

Table S2: Primers used for the complete sequencing of the CePV1 genome. The attachment position of each primer is based on the GenBank accession JQ744282

Primer sequence	genome position
E1endF 5'-CCTTGTCCCACCGATGAGAAT-3'	2340-2360
E1startR2 5'-GCACCTGAGATTCATCATCAC-3'	766-786
L1startR 5'-TATCTGGAAGTGCAAACTGA-3'	5931-5950
L1endF 5'-ACAGGCCAGATGCAGGTTCAT-3	6221-6241
L2endR 5'-CTCTGCTCACACCAATCCTAC-3'	5164-5184
L1endFII 5'-TCCTGGAGCACTGGGACAT-3'	6893-6911
E2E4startFII 5'-TCACCTTAGACACAACCTTGAAG-3'	3069-3091
L2startR 5'-CAGTATCTTATCTGCAACAGT-3'	4352-4372

Table S3: Scientific names, GENBANK accession numbers, and references of the genomic sequences of the animal hosts considered in the host-parasite interactions analyses.

Species	Source	GENBANK accession number	Reference
Odocoileus virginianus	mithocondrial genome bases 1-16480	JN632672	Hassanin,et al., 2012 C. R. Biol. 335 (1), 32-50
Alces alces	mithocondrial genome bases 1-16417	JN632595	Hassanin,et al., 2012 C. R. Biol. 335 (1), 32-50
Ovis aries	mithocondrial genome bases 1-16616	AF010406	Hiendleder et al., 1998 Anim. Genet. 29 (2), 116-122
Capreolus capreolus	mithocondrial genome bases 1-16358	JN632610	Hassanin,et al., 2012 C. R. Biol. 335 (1), 32-50
Bos taurus	mithocondrial genome bases 1 to 16338	AF492351	Hiendleder et al. 2008 Cytogenet. Genome Res. 120 (1-2), 150-156
Rangifer tarandus	mithocondrial genome bases 1 - 16362	AB245426	Wada et al. 2005 unpublished
Cervus elaphus	mithocondrial genome bases 1-16357	AB245427	Wada et al. 2005 unpublished

References

Ahola, H., P. Bergman, A.C. Ström, J. Moreno-Lopéz, and U. Pettersson. 1986. Organization and expression of the transforming region from the European elk papillomavirus (EEPV). Gene **50:**195–205.

Chen, E.Y., P.M. Howley, A.D. Levinson, and P.H. Seeburg. 1982. The primary structure and genetic organization of the bovine papillomavirus type 1 genome. Nature **299:**529–534.

Chen, Z., M. Schiffman, R. Herrero, R. Desalle, and R.D. Burk. 2007. Human papillomavirus (HPV) types 101 and 103 isolated from cervicovaginal cells lack an E6 open reading frame (ORF) and are related to gamma-papillomaviruses. Virology **360:**447–453.

Christensen, N.D., N.M. Cladel, C.A. Reed, and R. Han. 2000. Rabbit oral papillomavirus complete genome sequence and immunity following genital infection. Virology **269:**451–461.

Cole, S.T., and O. Danos. 1987. Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. Phylogeny of papillomaviruses and repeated structure of the E6 and E7 gene products. J. Mol. Biol. **193:**599-608.

Danos, O., M. Katinka, and M. Yaniv. 1982. Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among papovaviridae. EMBO J. **1:**231-236

Delius, H., and B. Hofmann. 1994. Primer-directed sequencing of human papillomavirus types. Curr. Top. Microbiol. Immunol. **186:**13-31.

Delius, H, B. Saegling, K. Bergmann, V. Shamanin, and E.M. de Villiers. 1998. The genomes of three of four novel HPV types, defined by differences of their L1 genes, show high conservation of the E7 gene and the URR. Virology. **240:**359-365.

Egawa, K., H. Delius, T. Matsukura, M. Kawashima, and E.M. de Villiers. 1993. Two novel types of human papillomavirus, HPV 63 and HPV 65: comparisons of their clinical and histological features and DNA sequences to other HPV types. Virology. **194:**789-799.

Erdelyi,K., Balint,A., Dencso,L., Dan,A. and Ursu,K. 2008. Characterisation of the first complete genome sequence of the roe deer (Capreolus capreolus) papillomavirus. Virus Res. **135 (2)**: 307-311

Favre, M, S. Obalek, S. Jablonska, and G. Orth. 1989. Human papillomavirus (HPV) type 50, a type associated with epidermodysplasia verruciformis (EV) and only weakly related to other EV-specific HPVs. J. Virol. **63:**4910.

Forslund, O., A. Antonsson, G. Higgins, H. Ly, H. Delius, A. Hunziker, and E.M. de Villier.

2003. Nucleotide sequence and phylogenetic classification of candidate human papilloma virus type 92. Virology. **312:**255-260
Fuchs, P.G., T. Iftner, J. Weninger, and H. Pfister. 1986. Epidermodysplasia verruciformis-associated human papillomavirus 8: genomic sequence and comparative analysis. J. Virol. **58:**626-634.
Ghim, S.J., A. Rector, H. Delius, J.P. Sundberg, A.B. Jenson, and M. Van Ranst. 2004. Equine papillomavirus type 1: complete nucleotide sequence and characterization of recombinant virus-like particles composed of the EcPV-1 L1 major capsid protein. Biochem. Biophys. Res. Commun. **324:**1108-1115.
Giri, I., O. Danos, and M. Yaniv. 1985. Genomic structure of the cottontail rabbit (Shope) papillomavirus. Proc. Natl. Acad. Sci. U.S.A. **82:**1580– 1584.
Groff, D.E., and W.D. Lancaster. 1985. Molecular cloning and nucleotide sequence of deer papillomavirus. J. Virol. **56:**85–91.
Herbst, L.H., J. Lenz, K. Van Doorslaer, Z. Chen, B.A. Stacy, J.F. Wellehan Jr., C.A. Manire, and R.D. Burk. 2009. Genomic characterization of two novel reptilian papillomaviruses, Chelonia mydas papillomavirus 1 and Caretta caretta papillomavirus 1. Virology **383:**131– 135.
Hatama,S., Nobumoto,K. and Kanno,T. 2008. Genomic and phylogenetic analysis of two novel bovine papillomaviruses, BPV-9 and BPV-10. J. Gen. Virol. **89**: 158-163
Hatama,S., Ishihara,R., Ueda,Y., Kanno,T. and Uchida,I. 2011. Detection of a novel bovine papillomavirus type 11 (BPV-11) using xipapillomavirus consensus polymerase chain reaction primers. **156(7):**1281-5 Arch. Virol.
Hirsch-Behnam, A., H. Delius, and E.M. de Villier. 1990. A comparative sequence analysis of two human papillomavirus (HPV) types 2a and 57. Virus Res. **18:**81-97.
Hirt, L., A. Hirsch-Behnam, and E.M. de Villiers. 1991. Nucleotide sequence of human papillomavirus (HPV) type 41: an unusual HPV type without a typical E2 binding site consensus sequence. Virus Res. **18:**1791-89.
Isegawa, N., K. Nakano, M. Ohta, H. Shirasawa, H. Tokita, and B. Simizu. 1994. Cloning and sequencing of the L1 gene of canine oral papillomavirus. Gene **146:**261–265.
Iwasaki, T., H. Maeda, M. Kameyama, M. Moriyama, S. Kanai, and T. Kurata. 1997. Presence of a novel hamster oral papillomavirus in dysplastic lesions of hamster lingual mucosa induced by application of dimethylbenzanthracene and excisional wounding: molecular cloning and complete nucleotide sequence. J. Gen. Virol. **78:**1087–1093.
Jarrett, W.F., M.S. Campo, B.W. O'Neil, H.M. Laird, and L.W. Coggins. 1984. A novel bovine papillomavirus (BPV-6) causing true epithelial papillomas of the mammary gland skin: a member of a proposed new BPV subgroup. Virology **136:**255–264.
Kremsdorf, D., M. Favre, S. Jablonska, S. Obalek, LA. Rueda, MA. Lutzner, C. Blanchet-Bardon, P.C. Van Voorst Vader, and G. Orth. 1984. Molecular cloning and characterization of the genomes of nine newly recognized human papillomavirus types associated with epidermodysplasia verruciformis. J. Virol. **52:**1013-1018
Lange,C.E., Tobler,K., Ackermann,M., Panakova,L., Thoday,K.L. and Favrot,C. 2009a. Three novel canine papillomaviruses support taxonomic clade formation. J. Gen. Virol. **90** (PT 11): 2615-2621
Lange, C.E., K. Tobler, T. Markau, Z. Alhaidari, V. Bornand, R. Stockli, M. Trussel, M. Ackermann, and C. Favrot, 2009b. Sequence and classification of FdPV2, a papillomavirus isolated from feline Bowenoid in situ carcinomas. Vet. Microbiol. **137:**60–65.
Matsukura, T., T. Iwasaki, and M. Kawashima. 1992. Molecular cloning of a novel human papillomavirus (type 60) from a plantar cyst with characteristic pathological changes. Virology **190:**561-564.
Moreno-Lopez, J., H. Ahola, A. Eriksson, P. Bergman, and U. Pettersson. 1987. Reindeer

papillomavirus transforming properties correlate with a highly conserved E5 region. J. Virol. **61:**3394–3400.
Müller, M., G. Kelly, M. Fiedler, L. Gissmann. 1989. Human papillomavirus type 48. J. Virol. **63:**4907-4908.
Nafz, J., K. Schafer, S.F. Chen, I.G. Bravo, M. Ibberson, I. Nindl, E. Stockfleth, and F. Rosl. 2008. A novel rodent papillomavirus isolated from anogenital lesions in its natural host. Virology **374:**186–197.
Ogawa, T., Y. Tomita, M. Okada, and H. Shirasawa. 2007. Complete genome and phylogenetic position of bovine papillomavirus type 7. J Gen Virol. **88:**1934-1938.
Ostrow, R.S., K.V. LaBresh, and A.J. Faras. 1991. Characterization of the complete RhPV 1 genomic sequence and an integration locus from a metastatic tumor. Virology **181:**424–429.
Patel, K.R., K.T. Smith, and M.S. Campo. 1987. The nucleotide sequence and genome organization of bovine papillomavirus type 4. J. Gen. Virol. **68:**2117–2128.
Rector, A., G.D. Bossart, S.J. Ghim, J.P. Sundberg, A.B. Jenson, and M. Van Ranst. 2004. Characterization of a novel close-to-root papillomavirus from a Florida manatee by using multiply primed rolling-circle amplification: Trichechus manatus latirostris papillomavirus type 1. J. Virol. **78:**12698–12702.
Rector, A., R. Tachezy, K. Van Doorslaer, T. MacNamara, R.D. Burk, J.P. Sundberg, and M. Van Ranst. 2005a. Isolation and cloning of a papillomavirus from a North American porcupine by using multiply primed rolling-circle amplification: the Erethizon dorsatum papillomavirus type 1. Virology **331**:449–456.
Rector, A., D.K. Van, M. Bertelsen, I.K. Barker, R.A. Olberg, P. Lemey, J.P. Sundberg, and M., Van Ranst. 2005b. Isolation and cloning of the raccoon (Procyon lotor) papillomavirus type 1 by using degenerate papillomavirus-specific primers. J. Gen. Virol. **86:**2029–2033.
Rector, A., S. Mostmans, K. Van Doorslaer, C.A. McKnight, R.K. Maes, A.G. Wise, M. Kiupel, and M. Van Ranst. 2006. Genetic characterization of the first chiropteran papillomavirus, isolated from a basosquamous carcinoma in an Egyptian fruit bat: the Rousettus aegyptiacus papillomavirus type 1. Vet. Microbiol. **117:**267–275.
Rector,A., Lemey,P., Tachezy,R., Mostmans,S., Ghim,S.J., Van Doorslaer,K., Roelke,M., Bush,M., Montali,R.J., Joslin,J., Burk,R.D.,
Jenson,A.B., Sundberg,J.P., Shapiro,B. and Van Ranst,M. 2007. Ancient papillomavirus-host co-speciation in Felidae. Genome Biol. **8** (4): 57
Rector, A., H. Stevens, G. Lacave, P. Lemey, S. Mostmans, A. Salbany, M. Vos, K. Van Doorslaer, S.J. Ghim, M. Rehtanz, G.D. Bossart,
A.B. Jenson, and M. Van Ranst. 2008. Genomic characterization of novel dolphin papillomaviruses provides indications for recombination within the Papillomaviridae. Virology **378:**151–161.
Rehtanz, M., S.J. Ghim, A. Rector, M. Van Ranst, P.A. Fair, G.D. Bossart, and A.B. Jenson. 2006. Isolation and characterization of the first American bottlenose dolphin papillomavirus: Tursiops truncatus papillomavirus type 2. J. Gen. Virol. **87:**3559–3565.
Salmon, J., M. Nonnenmacher, S. Cazé, P. Flamant, O. Croissant, G. Orth, and F. Breitburd. 2000. Variation in the nucleotide sequence of cottontail rabbit papillomavirus a and b subtypes affects wart regression and malignant transformation and level of viral replication in domestic rabbits. J. Virol. **74:**10766-10777.
Scase T, Brandt S, Kainzbauer C, Sykora S, Bijmholt S, Hughes K, Sharpe S, Foote A. 2010. Equus caballus papillomavirus-2 (EcPV-2): an infectious cause for equine genital cancer? Equine Vet J. **42**(8):738-45
Seedorf, K, G. Krämmer, M. Dürst, S. Suhai, and WG. Röwekamp. 1985. Human papillomavirus type 16 DNA sequence. Virology **145:**181- 185.
Scheurlen, W., L. Gissmann, G. Gross, and H. zur Hausen. 1986. Molecular cloning of two new HPV types (HPV 37 and HPV 38) from a keratoacanthoma and a malignant melanoma. Int. J. Cancer **37:**505-510.
Schulz, E., M. Gottschling, I.G. Bravo, U. Wittstatt, E. Stockfleth, and I. Nindl. 2009. Genomic characterization of the first insectivoran papillomavirus reveals an unusually long, second non-coding region and indicates a close relationship to betapapillomavirus. J. Gen. Virol. **90:**626– 633.
Schulz,E., Gottschling,M., Wibbelt,G., Stockfleth,E. and Nindl,I. 2009b. Isolation and genomic characterization of the first Norway rat (Rattus norvegicus) papillomavirus and its phylogenetic position within Pipapillomavirus, primarily infecting rodents. J. Gen. Virol. **90** (PT 11): 2609-2614
Schwarz, E., M. Dürst, C. Demankowski, O. Lattermann, R. Zech, E. Wolfsperger, S. Suhai, H. zur

Hausen. 1983. DNA sequence and genome organization of genital human papillomavirus type 6b. EMBO J. **2:**2341-2348.
Stevens, H., A. Rector, K. Van Der Kroght, and M. Van Ranst. 2008a. Isolation and cloning of two variant papillomaviruses from domestic pigs: Sus scrofa papillomaviruses type 1 variants a and b. J. Gen. Virol. **73:**2475–2481.
Stevens, H., A. Rector, M.F. Bertelsen, P.S. Leifsson, and M. Van Ranst. 2008b. Novel papillomavirus isolated from the oral mucosa of a polar bear does not cluster with other papillomaviruses of carnivores. Vet. Microbiol. **129:**108–116.
Tachezy, R., G. Duson, A. Rector, A.B. Jenson, J.P. Sundberg, and M. Van Ranst. 2002a. Cloning and genomic characterization of Felis domesticus papillomavirus type 1. Virology **301:**313–321.
Tachezy, R., A. Rector, M. Havelkova, E. Wollants, P. Fiten, G. Opdenakker, B. Jenson, J. Sundberg, and M. Van Ranst. 2002b. Avian papillomaviruses: the parrot Psittacus erithacus papillomavirus (PePV) genome has a unique organization of the early protein region and is phylogenetically related to the chaffinch papillomavirus. BMC Microbiol. **2:**19–27.
Tan, C.H., R. Tachezy, M. Van Ranst, S.Y. Chan, H.U. Bernard, and R.D. Burk. 1994. The Mastomys natalensis papillomavirus: nucleotide sequence, genome organization, and phylogenetic relationship of a rodent papillomavirus involved in tumorigenesis of cutaneous epithelia. Virology
198:534–541.
Terai, M., R. DeSalle, and R.D. Burk. 2002. Lack of canonical E6 and E7 open reading frames in bird papillomaviruses: Fringilla coelebs papillomavirus and Psittacus erithacus timneh papillomavirus. J. Virol. **76:**10020–10023.
Tobler, K., C. Favrot, G. Nespeca, M. Ackermann. 2006. Detection of the prototype of a potential novel genus in the family Papillomaviridae in association with canine epidermodysplasia verruciformis. J. Gen. Virol. **87:**3551–3557.
Tobler, K., C. Lange, D.N. Carlotti, M. Ackermann, and C. Favrot. 2008. Detection of a novel papillomavirus in pigmented plaques of four pugs. Vet. Dermatol. **19:**21–25.
Tomita, Y., I. Literak, T. Ogawa, Z. Jin, and H. Shirasawa. 2007. Complete genomes and phylogenetic positions of bovine papillomavirus type 8 and a variant type from a European bison. Virus Genes **3:**243–249.
Van Bressem,M.F., Cassonnet,P., Rector,A., Desaintes,C., Van Waerebeek,K., Alfaro-Shigueto,J., Van Ranst,M. and Orth,G. 2007. Genital warts in Burmeister's porpoises: characterization of Phocoena spinipinnis papillomavirus type 1 (PsPV-1) and evidence for a second, distantly related PsPV. J. Gen. Virol. **88** (PT 7): 1928-1933
Van Doorslaer, K., A. Rector , P. Vos , and M. Van Ranst. 2006. Genetic characterization of the Capra hircus papillomavirus: a novel close-toroot artiodactyl papillomavirus. Virus Res. **118:**164-169. Van Doorslaer,K., Sidi,A.O., Zanier,K., Rybin,V., Deryckere,F., Rector,A., Burk,R.D., Lienau,E.K., van Ranst,M. and Trave,G. 2009. Identification of unusual E6 and E7 proteins within avian papillomaviruses: cellular localization, biophysical characterization, and phylogenetic analysis. J. Virol.
83 (17): 8759-8770
Van Ranst, M., A. Fuse, H. Sobis, W. De Meurichy, S.M. Syrjanen, A. Billiau, and G. Opdenakker. 1991. A papillomavirus related to HPV type 13 in oral focal epithelial hyperplasia in the pygmy chimpanzee. J. Oral Pathol. Med. **20:**325–331.
Vasiljević, N., K. Hazard, L. Eliasson, H. Ly, A. Hunziker, E.M. de Villiers, B. Norrild, J. Dillner, and O. Forslund. 2007. Characterization of two novel cutaneous human papillomaviruses, HPV93 and HPV96. J. Gen. Virol. **88:**1479-1483.
Wood,C.E., Tannehill-Gregg,S.H., Chen,Z., Doorslaer,K., Nelson,D.R., Cline,J.M. and Burk,R.D. 2011. Novel betapapillomavirus associated with hand and foot papillomas in a cynomolgus macaque Vet. Pathol. **48** (3): 731-736
Yuan, H., S. Ghim, J. Newsome, T. Apolinario, V. Olcese, M. Martin, H. Delius, P. Felsburg, B. Jenson, and R. Schlegel. 2007. An epidermotropic canine papillomavirus with malignant potential contains an E5 gene and establishes a unique genus. Virology **359:**28–36.
Zachow, K.R., R.S. Ostrow, and A.J. Faras. 1987. Nucleotide sequence and genome organization of human papillomavirus type 5. Virology
158:251-254.

Zhu,W., Dong,J., Shimizu,E., Hatama,S., Kadota,K., Goto,Y. and Haga,T. 2012. Characterization of novel bovine papillomavirus type 12 (BPV-12) causing epithelial papilloma. Arch. Virol. **157** (1): 85-91

Paper V

Papillomavirus in healthy skin and mucosa of Alpine wild ruminants

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Abstract

An investigation on healthy skin and mucosal specimens of wild ruminants shot in the Italian Alps in Belluno province has been performed. We report for first time the presence of BPV-2 DNA in the healthy skin of wild ruminants and the co-infection of BPV-1 and CePV-1 in a healthy red deer. Besides the *Delta* PVs, already known to infect species different from their specific host, a cross-infection of BPVs belonging to *Xi*-genus, found as single infection and also in association with *Delta* BPV types 1 and 2 has been demonstrated confirming that host tropism of PVs is not as species-specific as previously thought. Our results suggest that subclinical infections could be linked to the presence of domestic ruminants sharing the same habitat with wild species and that the latter may act as reservoir for domestic species' PVs.

Keywords: bovidae, cervidae, co-infection, mucosa, papillomavirus, skin

Introduction

The family *Papillomaviridae* comprises over 32 genera infecting animals (Rector and Van Ranst 2013). In ruminants papillomaviruses (PVs) belonging to genera *Delta* (BPV-1, 2, 13, BgPV-1, CcaPV-1, OaPV-1, 2, OvPV-1, AaPV-1*)*, *Epsilon* (BPV-5, 8), *Xi* (BPV3, 4, 6, 9, 10, 11, 12), *Phi* (ChPV-1), *Dyolambda* (OaPV-3, RrupPV-1) and *Dyoxi* (BPV-7), induce hyperplastic benign lesions of both cutaneous and mucosal epithelia that generally regress. The disease may occasionally persist with the possibility to evolve into cancer in the presence of environmental carcinogenic co-factors.

Papillomaviruses are known to be highly species specific, with the exception of Bovine papillomavirus types 1 and 2 (BPV-1, 2), and more recently BPV-13 (Lunardi et al. 2013). These viruses belong to the genus *Delta* papillomavirus and they are all causative agents of equine sarcoid in horses and donkeys (Lancaster et al. 1979; Amtmann 1980; Lunardi et al. 2013) even if they were also detected in wild species (Löhr et al. 2005; Literák et al. 2006; Kidney and Berrocal, 2008; Silvestre et al. 2009; van Dyk et al. 2011). PVs infect the basal layer of cutaneous or mucosal epithelial cells of vertebrates; infections occur via microlesions of proliferating basal layer cells. Virus spread occurs by virus release from the surface of warts and papillomatous lesions which frequently contain large quantities of viral particles within their superficial differentiated layers. Following infection, there are two potential outcomes: a clinically apparent lesion may form or, the onset of a clinically silent infection. The latter may or may not involve the completion of the full productive virus life cycle, where new virions are not formed representing a form of viral latency (Maglennon and Doorbar, 2012) with a possible reactivation under conditions of immunosuppression. The presence of the virus is well documented in healthy skin of humans and different other animal species (Antonsson and Hansson, 2002; Antonsson and McMillan 2006) as a presumed commensal agent (Antonsson et al. 2003). Papillomovavirus infections have been recently reported in a red deer (*Cervus elaphus)* from the Alps of Stelvio Park (Scagliarini et al. 2013) and from a

chamois (*Rupicapra rupicapra)* from Val d'Aosta Alps (Mengual Chulià, 2014). Despite a very limited morbidity, these two cases proved the circulation of papillomaviruses among Alpine wild ruminants leading to speculate a role of reservoir for these animal species. This study was aimed at investigating the distribution of papillomaviruses in Alpine wild ruminants, to address this issue, PCR assays with previously published primer combinations targeting the L1 open reading frame (ORF) were performed.

Materials and Methods

A total of 112 samples collected from 32 deer (*Cervus elaphus*), 13 chamois (*Rupicapra rupicapra*), 5 roe deer (*Capreolus capreolus*) and 6 mouflons (*Ovis musimon*) shot within the faunal control program were sampled. Skin from coronary band and labial mucosa were harvested, and each subject was identified with the same serial number and a letter depending on the site of sampling (Z= coronary band; B= labial mucosa). None of the subjects showed any lesion referable to epitheliotropic viruses neither on the sampling site nor on the rest of the body. After collection, all samples were conserved -80°C until processed.

DNA was extracted from samples with Nucleo Spin Tissue (Macherey-Nagel). The cytochrome C oxidase subunit I (COX I) gene, was amplified (data not shown) and used as indicator of nucleic acid extraction and quality of samples.

PCR reactions were performed with "*Taq* PCR Master Mix Kit" (Qiagen) on a final volume of 25µl containing 1X PCR buffer, 5 µl of Q-solution, 0,8 mM deoxynucleotide mix (Thermo Scientific), 0,4 µM of sense and antisense primer, 2,5 µl of DNA template and 0,65 units of Taq DNA polymerase. The amplification program consisted of an initial denaturation step of 94°C for 5 min followed by 40 thermal cycles [94°C 30 sec; AT 30 sec; 72°C 1 min] and a final elongation step at 72°C for 5 min.

All samples have been amplified with BPV types 1 and 2 (*Delta*) and BPV *Xi*-genus specific primer pair (BPV310F/R AT: 52°C), and if found positive for the latter, type specific (BPV-3,

6, 9, 10) amplification was performed (BPV3/6F AT: 52°C; BPV9F/R AT: 60°C; BPV10F/R AT: 60°C. Mouflons were also tested with Ocp1s/2r primer pair (kindly provided by Alberti A. University of Sassari) targeting OaPV-1, 2 and 3 (*Ovis aries*) as well as ChPV-1 (*Capra hircus*). CePV-1 (*Cervus elaphus*) primer pair (L1 end F2/E1 start R2 AT 55°C) was used to screen all red deer samples (Table 1). Each reaction included a no template control (NTC), an appropriate positive control consisting in a previously sequenced PV type. All standard precautions adhering to strict laboratory practices were followed to prevent any contamination. Different steps (DNA extraction, sample preparation, amplification) were performed in strictly separated rooms.

Nineteen randomly selected products arising from 12 5'B1/2-E5- 3'B1/2-E5, 6 BPV310f/r and 1 L1 end F2/E1 start R2 were sequenced. Subsequent Sanger sequencing was performed on both strands of the amplified DNA (ABI Prism 3100 Genetic Analyzer, Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and chromatograms were assembled using 4Peaks 1.7.1.

Results

PV DNA was detected in around 70% of the tested animals (Table 2): in the 37.5 % of skin samples whose mucosal samples were found negative, in the 10.7 % of mucous samples whose skin samples were found negative and in the 21,42 % of samples from both sampling sites.

In particular BPV-1/2 DNA was detected in 19/32 red deer, 7/13 chamois, 3/5 roe deer and 5/6 mouflons (17 mucosal samples and 28 skin samples), five deer and three chamois tested positive in both compartments.

Random sequencing of E5 amplicons demonstrated the presence of BPV types 1 and 2, as well as BPV-1 EqSarc variant (GenBank accession number: JX678969), that shows a single

synonymous point mutation (A→60G) to date identified in BPV-1 amplified from lesions of sarcoid bearing-horses only.

Identity among BPV-2 amplicons was 100%, among BPV-1 ranged between 99,3%- 100%, while EqSarc sequences ranged between 99,3% and 98,5%.

Xi-genus BPVs were detected in 4/13 chamois, 3/5 roe deer, 3/32 red deer and 1/6 mouflons, in this case no subject tested positive both in mucosa and skin. Sequencing was performed on all amplicons, but clear DNA sequences were obtained only for samples 32B and 7Z. The latter demonstrated 74% identity with BPV-10 (GenBank accession number: KF017607), while for sample 32B the identity was 74% with BPV-12 (GenBank accession number: JF834524), 73% with BPV-6 (GenBank accession number: AB845589), and BPV3 with 72.89% identity (GenBank gi|23216976). Further investigations with specific primer pair aimed at the identification of BPV-3, 6, 9, 10 between *Xi*-genus positive samples were performed, showing that all the samples were not bearing this viral types.

Co-infections between *Delta* and *Xi* genera were identified in three red deer and three chamois.

No ovine or caprine PVs were detected neither in mouflon nor in chamois' samples.

One sample only (15Z), obtained from skin of a red deer, showed positivity for CePV; sequencing revealed 100% identity with CePV-1(GenBank JQ744282). The same sample tested positive also for BPV-1.

Discussion

Ubiquity and impressive multiplicity of PV genotypes amongst healthy skin, have been documented both in humans (Boxman et al. 1997; Astori et al. 1998; Antonsson et al. 2000) and in animals (Antonsson and Hansson, 2002; Lange et al. 2011; Silva et al. 2013), to such an extent that papillomaviruses have been described as the first viral skin commensals (Antonsson et al. 2000).

Species-specific papillomaviruses have been already identified in skin tumors of several wild ruminants such as white-tailed deer (OvPV-1), reindeer (RtPV-1), European elk (EePV-1), roe deer (CcPV-1) and chamois (RruPV-1) in Europe and North America (Moreno-Lopez et al.1981; Stenlund et al. 1983; Groff and Lancaster, 1985; Erdélyi et al. 2008; Mengual Chulià et al. 2014). Among healthy subjects belonging to *Cervidae*, a European elk tested positive for specific PVs.

Antonsson and Colleague (2002) performed analysis on healthy skin from various non-human vertebrate species, detecting specific European elk (*Alces alces*) PVs (EEPV plus three putative new EEAA1 to–3) in their host. Mengual Chulià (2014) performed an investigation on different species of the *Bovidae* and *Cervidae* families and detected DNA belonging to PVs infecting cattle, cat, ovine and human, but no species-specific PVs were amplified.

Xi genus comprises pure epitheliotropic viruses affecting cattle; *Delta* genus comprehends Artyodactila PVs, including BPV types 1 and 2, infecting both epithelia and underlying derma's cells and therefore known as fibropapillomaviruses. BPV1 and 2 are the only PVs able to infect cross-species causing common skin tumours of equids termed equine sarcoid, but cross-species infection of BPV has also been reported in other animal species.

In this study the positivity for BPV DNA in nonlesional skin and mucosa of wild ruminants was shown. Red deer were the most represented among the tested species (32/56), but surprisingly only one CePV, in association with BPV-1, was identified, resembling a co-infection already described by Scagliarini and colleagues (2013). The Authors identified both viruses in a diseased red deer from the Stelvio Park and quantitative analysis demonstrate that the CePV-1 DNA yield was four logarithms higher than BPV-1 DNA, confirming that the clinical lesions were ascribable to a productive CePV1 infection.

Among *Delta* types, the sequenced E5 gene revealed the presence of BPV-1, 2 and BPV-1 EqSarc1 E5 gene isolate (GenBank accession number JX678969), which presents a single nucleotide mutation that was suggested to increase codon usage in equine cells (Chambers et

al. 2003). It is noteworthy that to date this mutation has been detected in sarcoid-bearing horses only but not in cattle, in addition we report the presence of Eqsarc1 variant in species not belonging to the *Equidae* family. This finding might suggest an "equine adaptation" of the virus variant (Nasir and Campo, 2008; Yuan et al. 2007), as this is known to cause lesion in horses, but it was also demonstrated to be capable to sub-clinically infect other species. The ability of Eqsarc1 variant to asymptomatically infect other species, suggests that wild subjects could be the reservoir hosts of this particular PV, and equine sarcoids could be the result of repeated cross-species infection (Trewby et. al 2014) by an ancient bovine PV subsequently adapted to both *Bovidae* and *Cervidae* . A similar situation has been described in cats, where the *Delta* FeSarPV causes sarcoids, but is also asymptomatically present in bovines, and therefore a cross-species infection has been suggested (Munday et al. 2010). How the virus can spread from non-productive lesions such as sarcoid and whether infection is productive in different species is currently unclear even if Brandt el al (2008) showed the presence of viral particle precursors, composed by BPV-1 DNA in complex with the L1 capsid protein, in sarcoid samples. No sheep and goats'specific PVs were detected, despite to the fact that the presence of these domestic species use to share grazing areas with wild ruminats from spring until autumn. This may suggest a low incidence of infection in domestics, or that PVs of sheep and goats known to date might preferentially infect other anatomical sites.

Roe deer also tested negative for specific PVs, this further confirms previous findings of an extensive investigation performed on the European wildlife disease databases that showed that the presence of roe deer fibropapillomatosis had been never detected in many countries such as France, Sweden, Germany or United Kingdom. To date, roe deer fibropapillomatosis has been documented only in Hungary, this could be linked to the genetic differentiation pattern of European roe deer populations or to peculiarities of deer habitat use and geography (Erdélyi et al. 2009).

Type-specific primer pair didn't succeed to amplify any BPV belonging to the *Xi*-genus despite the positivity obtained with the generic genus specific primer pair, this result could be ascribed to sequence variability between the PVs circulating in Italy, as it has been previously demonstrated in Brazil (Carvalho et al. 2012).

Co-infections are common in humans and have been already documented in cutaneous lesions of bovines in different countries, but no information is available about the consequences in animals. This study demonstrated the presence of co-infections between BPVs belonging to *Delta* and *Xi* genera in the healthy skin and mucosa of chamois and deer. The frequent identification of *Delta* with *Xi* BPVs has also been reported in lesions of Brazilian and Indian cattle (Claus et al. 2009b; Carvalho et al. 2012; Kumar et al. 2013), suggesting an advantageous interaction between these viruses, or that the acquisition of one type of PV may facilitate the acquisition of another type.

Co-infections with two to three HPV types have been also identified in the vagina as well as in the skin of more than 50% of healthy subjects (Ma et al. 2014). Our findings show that this situation is common also in wild animals and may reflect the overall situation in domestic species. The presence of PV DNA in the skin and mucosa of wild ruminants might suggest that these compartments may represent a reservoir for bovine papillomaviruses.

It remains to be determined whether the virus can be spread by the host or not; transmission between wild and domestic species is expected to happen via an indirect way, as strict contact between subjects is more uncommon. Frequently, wild ruminants share their habitat and forage with domestic ruminant species (Ruiz de Ybànez et al. 2009). Under these conditions, transmission of infective agents including PVs among domestic and wild animals might be enhanced (Daszak and Cunningham, 2000). Due to livestock summer pasturing, this situation is very common on the Italian Alps and arthropod vectors may be involved in virus dissemination as PCR analysis already demonstrated the presence of the same variants of BPV-1 in equine sarcoids and in different flies' species (Finlay et al. 2009). A similar role for

arthropods has also been suggested in the ecology of CcPV1 (Erdélyi et al. 2009). Stool samples of different animals tested positive for the presence of specific PVs (Marshall et al. 1984; Gartrell et al. 2009; Phan et al. 2011). Recently Di Bonito and colleagues (2015) have demonstrated the elimination of both mucosal end epithelial PVs with feces in humans, confirming that epitheliotropic viruses can find their way into sewage (Cantalupo et al. 2011) not only through the washing of skin and mucous membrane, highlighting the possibility of a waterborne transmission. Skin from the coronary band and buccal mucosa are both prone to traumatic microlesion, in addition these are the body regions in closer contact with ground. Bovine papillomavirus type 1 has been shown to survive desiccation in cell extracts for up to 7 days (Roden et al. 1997), and this might increase infection rates in grazing subjects.

In conclusion, this study confirms that host tropism in PVs is not as species-specific as previously thought. Althought the presence of the genome of the *Delta* PV BPV-1 was already reported in different animal species, this is first identification of BPV-2 and BPV-1 and CePV-1 co-infection in the healthy skin of wild ruminants. To our knowledge this is also the first report of cross-infections of BPVs belonging to *Xi*-genus found as single infection and in association with *Delta* BPV types 1 and 2. Taken all together, our results strongly suggest the reservoir role of wild ruminants belonging to the *Bovidae* and *Cervidae* families for domestic ruminant PVs.

Literature Cited

Amtmann E, Müller H, Sauer G. 1980. Equine connective tissue tumors contain unintegrated bovine papilloma virus DNA. *Journal of Virology* 35 (3):962-964.

Antonsson A, Erfurt C, Hazard K, Holmgren V, Simon M et al. 2003. Prevalence and type spectrum of human papillomaviruses in healthy skin samples collected in three continents. *J Gen Virol* 84:1881-1886

Antonsson A, Forslund O, Ekberg H, Sterner G, Hansson BG. 2000. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. *J Virol* 74:11636–11641

Antonsson A, Hansson BG. 2002. Healthy skin of many animal species harbors papillomaviruses which are closely related to their human counterparts. *J Virol* 76 (24):12537-12542

Antonsson A, McMillan NA. 2006. Papillomavirus in healthy skin of Australian animals. *J Gen Virol*. 87(Pt 11):3195-200.

Astori G, Lavergne D, Benton C, Höckmayr B, Egawa K, Garbe C, de Villiers EM. 1998. Human papillomaviruses are commonly found in normal skin of immunocompetent hosts. *J Invest Dermatol.* 110(5):752-5

Boxman, I. L. Berkhout, R. J. Mulder, L. H. Wolkers, M. C. Bouwes Bavinck, J. N. Vermeer, B. J. & ter Schegget, J. 1997. Detection of human papillomavirus DNA in plucked hairs from renal transplant recipients and healthy volunteers. *J Investig Dermatol* 108, 712–715

Brandt S, Haralambus R, Shafti-Keramat S, Steinborn R, Stanek C, Kirnbauer R. 2008. A subset of equine sarcoids harbours BPV-1 DNA in a complex with L1 major capsid protein. *Virology.* 375(2):433-41

Brandt S, Apprich V, Hackl V, Tober R, Danzer M, Kainzbauer C, Gabriel C, Stanek C. Kofler J. 2011. Prevalence of bovine papillomavirus and Treponema DNA in bovine digital dermatitis lesions. *Vet. Microbiol.* 148(2-4): 161-167.

Cantalupo PG, Calgua B, Zhao G, Hundesa A, Wier AD, Katz JP, Grabe M, Hendrix RW, Girones R, Wang D, Pipas JM. 2011. Raw sewage harbors diverse viral populations. *MBio*. 2(5)

Carvalho CC, Batista MV, Silva MA, Balbino VQ, Freitas AC. 2012. Detection of bovine papillomavirus types, co-infection and a putative new BPV11 subtype in cattle. *Transbound Emerg Dis* 59(5):441-7

Chambers G, Ellsmore VA, O'Brien PM, Reid SW, Love S, Campo MS, Nasir L. 2003. Sequence variants of bovine papillomavirus E5 detected in equine sarcoids. *Virus Res*. 96(1-2):141-5

Claus MP, Lunardi M, Alfieri AA, Otonel AA, Ferracin LM, Fungaro MHP, Alfieri AF. 2009b. A bovine teat papilloma specimen harboring Deltapapillomavirus (BPV-1) and Xipapillomavirus (BPV-6) representatives. *Braz Arch Biol Technol* 52 (spe):87-91.

Daszak P, Cunningham AA. 2000. More on the ecological impact of fungal infections on wildlife populations. *Parasitol Today* 16:404–405.

Di Bonito P, Della Libera S, Petricca S, Iaconelli M, Sanguinetti M, Graffeo R, Accardi L, La Rosa G. 2015. A large spectrum of alpha and beta papillomaviruses are detected in human stool samples. *J Gen Virol*. 96(Pt 3):6

Erdélyi K, Bálint A, Dencso L, Dán A, Ursu K. 2008. Characterisation of the first complete genome sequence of the roe deer (Capreolus capreolus) papillomavirus. *Virus Res*135(2):307-11

Erdélyi K, Dencso L, Lehoczki R, Heltai M, Sonkoly K, Csányi S, Solymosi N. 2009. Endemic papillomavirus infection of roe deer (Capreolus capreolus). *Vet Microbiol* 2;138(1-2):20-6

Finlay M, Yuan Z, Burden F, Trawford A, Morgan IM, Campo MS, Nasir L. 2009. The detection of Bovine Papillomavirus type 1 DNA in flies. *Virus Res* 144(1-2):315-7

Gartrell BD, Morgan KJ, Howe L, Munday JS, McFadden AM. 2009. Cloacal papillomatosis in the absence of herpesvirus and papillomavirus in a sulphur-crested cockatoo (Cacatua galerita). *N Z Vet J* 57(4):241-3

Groff DE, Lancaster WD. 1985. Molecular cloning and nucleotide sequence of deer papillomavirus. *J Virol. Oct* 56(1):85-91

Kidney BA, Berrocal A. 2008. Sarcoids in two captive tapirs (*Tapirus bairdii*): clinical, pathological and molecular study. *Vet Dermatol* 19 (6):380-384.

Kumar P, Jangir BL, Saikumar G, Somvanshi R. 2013. Co-infection of bovine papillomavirus type-1 and -10 in teat warts of dairy cattle. *Vet Med-Czech* 58 (12):605-608

Lancaster WD, Theilen GH, Olson C. 1979. Hybridization of bovine papilloma virus type 1 and type 2 DNA to DNA from virus-induced hamster tumors and naturally occurring equine tumors. *Intervirology*; 11 (4):227-233.

Lange CE, Zollinger S, Tobler K, Ackermann M, Favrot C. 2011. Clinically healthy skin of dogs is a potential reservoir for canine papillomaviruses. *J Clin Microbiol*. 49(2):707-9

Literák I, Tomita Y, Ogawa T, Shirasawa H, Šmíd B, Novotný L, Adamec M. 2006. Papillomatosis in a european bison. *J Wildl Dis* 42 (1):149-153

Löhr CV, Juan-Sallés C, Rosas-Rosas A, Parás García A, Garner MM, Teifke JP. 2005. Sarcoids in captive zebras (*Equus burchellii*): association with bovine papillomavirus type 1 infection. *J Zoo Wildl Med* 36 (1): 74-81

Lunardi M, de Alcantara BK, Otonel RAA, Rodrigues WB, Alfieri AF, Alfieri AA. 2013. Bovine papillomavirus type 13 DNA in equine sarcoids. *J Clin Microbiol* 51 (7), 2167-2171.

Ma Y, Madupu R, Karaoz U, Nossa CW, Yang L, Yooseph S, Yachimski PS, Brodie EL, Nelson KE, Pei Z. 2014. Human papillomavirus community in healthy persons, defined by metagenomics analysis of human microbiome project shotgun sequencing data sets. *J Virol*. 88(9):4786-97

Maglennon GA, Doorbar J. 2012. The biology of papillomavirus latency. *Open Virol J* 6:190-7

Marshall JA, Healey DS, Studdert MJ, Scott PC, Kennett ML, Ward BK, Gust ID. 1984. Viruses and virus-like particles in the faeces of dogs with and without diarrhoea. *Aust Vet J*. 61(2):33-8

Mengual-Chuliá B, Domenis L, Robetto S, Bravo IG. 2014. A novel papillomavirus isolated from a nasal neoplasia in an Italian free-ranging chamois (Rupicapra r. rupicapra). *Vet Microbiol*. 172(1-2):108-19

Mengual-Chuliá B, García-Pérez R, Gottschling M, Nindl I, Bravo IG. 2012. Novel animal papillomavirus sequences and accurate phylogenetic placement. *Mol Phylogenet Evol*. 65(3):883-91

Moreno-Lopez J, Pettersson U, Dinter Z, Philipson L. 1981. Characterization of a papilloma virus from the European elk (EEPV). *Virology*. 30;112(2):589-95

Munday JS, Knight CG. 2010. Amplification of feline sarcoid-associated papillomavirus DNA sequences from bovine skin. *Vet Dermatol*. 21(4):341-4

Nasir L, Campo MS. Bovine papillomaviruses: their role in the aetiology of cutaneous tumours of bovids and equids. 2008. *Vet Dermatol* 19(5):243-54

Phan TG, Kapusinszky B, Wang C, Rose RK, Lipton HL, Delwart EL. 2011. The fecal viral flora of wild rodents. *PLoS Pathog*. 7(9):e1002218

Rector A, Van Ranst M. 2013. Animal papillomaviruses. *Virology* 445(1-2):213-23

Roden RB, Lowy DR, Schiller JT. 1997. Papillomavirus is resistant to desiccation. *J Infect Dis.* 176(4):1076-9

Ruiz De Ybanez M R, Martinez-Carrasco C, Alonso FD, Leonn L. 2009. Lungworm infection of wild ruminants in the Sierras de Cazorla, Segura and Villas Natural Park (Jaén, España). *Acta Parasitol Port* 16:184–185

Scagliarini A, Gallina L, Battilani M, Turrini F, Savini F, Lavazza A, Chiari M, Coradduzza E, Peli A, Erdélyi K, Alberti A. 2013. Cervus elaphus papillomavirus (CePV1): new insights on viral evolution in deer. *Vet Microbiol*. 30;165(3-4):252-9

Silva MA, Altamura G, Corteggio A, Roperto F, Bocaneti F, Velescu E, Freitas AC, Carvalho CC, Cavalcanti KP, Borzacchiello G. 2013. Expression of connexin 26 and bovine papillomavirus E5 in cutaneous fibropapillomas of cattle. *Vet J*. 195(3):337-43.

Silvestre O, Borzacchiello G, Nava D, Iovane G, Russo V, Vecchio D, D'Ausilio F, Gault EA, Campo MS, Paciello O. 2009. Bovine papillomavirus type 1 DNA and E5 oncoprotein expression in water buffalo fibropapillomas. *Vet Pathol* 46(4):636-41

Stenlund A, Moreno-Lopez J, Ahola H, Pettersson U. 1983. European elk papillomavirus: characterization of the genome, induction of tumors in animals, and transformation in vitro. *J Virol* 48(2):370-6

Trewby H, Ayele G, Borzacchiello G, Brandt S, Campo MS, Del Fava C, Marais J, Leonardi L, Vanselow B, Biek R, Nasir L. 2014. Analysis of the long control region of bovine papillomavirus type 1 associated with sarcoids in equine hosts indicates multiple cross-species transmission events and phylogeographical structure. *J Gen Virol* 95(Pt 12):2748-56

van Dyk E, Bosman AM, van Wilpe E, Williams JH, Bengis RG, van Heerden J, Venter EH. 2011. Detection and characterization of papillomavirus in skin lesions of giraffe and sable antelope in South Africa. *J. S. Afr. Vet. Assoc*; 82 (2):80-85.

Yuan Z, Philbey AW, Gault EA, Campo MS, Nasir L. 2007. Detection of bovine papillomavirus type 1 genomes and viral gene expression in equine inflammatory skin conditions. *Virus Res* 124(1-2):245-9

Table 1- Primer pair for amplification of different PV types and genera

Primer pair	**Sequence**	**Type of virus**	**Genus**	**Reference**
5'B1/2-E5-	F 5'-CACTACCTCCTGGAATGAACATTTCC -3'	BPV-1, 2	*Delta*	Brandt et al.
3'B1/2-E5	R 5'-CTACCTTWGGTATCACATCTGGTGG -3'			2008
BPV310f	F 5' - RACACGGATGABTTTGTYACWCGMAC -3'	BPV-3, 4, 6, 9, 10, 11,	*Xi*	Brandt et al.
BPV310r	R 5' - CCCARTGYTCHCCWTCRCARGG -3'	12		2011
BPV10F	F 5'- AAGGCATTTGTGGTCTCGAGG -3'	BPV-10	*Xi*	Carvalho et al.
BPV10R	R 5'- CTAAAGAACCACTTGGAGTGCC -3'			2012
BPV3/6 F	F 5'- CAGTCAATTGCAACTAGATGCC -3'	BPV-3, 6	*Xi*	Carvalho et al.
BPV3/6 R	R 5'- GGCTGCTACTTTCAAAAGTGA -3'			2012
BPV9F	F 5'- AAAGAGCAAATCGGGAGCACC -3'	BPV-9	*Xi*	Carvalho et al.
BPV9R	R 5'- AACTAATGACCCACTAGGGCTCC -3'			2012
L1 end FII	F 5'- TCCTGGAGCACTGGGACAT -3'	CePV-1	*Delta*	Scagliarini et al
E1 start R2	R 5'-GCACCTGAGATTCATCATCAC -3'			2013

Table 2-Papillomavirus types detected in healthy skin and mucosa of wild ruminants.

Species	ID	Mucosa	Skin
Chamois	1	-	-
Chamois	2	-	-
Chamois	3	ξ	-
Red deer	4	δ and ξ	-
Red deer	5	-	δ
Red deer	6	-	δ
Red deer	7	δ	δ and ξ
Red deer	8	-	-
Red deer	9	-	-
Red deer	10	-	-
Red deer	11	δ	-
Red deer	12	-	-
Red deer	13	-	-
Red deer	14	-	-
Red deer	15	-	δ and CePV-1
Red deer	16	-	δ
Chamois	17	δ	δ and ξ
Chamois	18	-	-
Red deer	19	δ	δ
Red deer	20	δ	δ
Red deer	21	-	-
Chamois	22	-	-
Red deer	23	-	δ
Chamois	24	-	δ
Red deer	25	-	δ
Chamois	26	-	δ
Red deer	27	δ	ξ
Red deer	28	-	-
Red deer	29	-	δ
Chamois	30	δ	δ
Red deer	31	δ	δ
Chamois	32	δ and ξ	δ
Red deer	33	-	δ
Red deer	34	-	δ
Red deer	35	-	-
Chamois	36	-	-
Chamois	37	δ and ξ	-
Chamois	38	-	δ
Red deer	39	-	δ
Red deer	40	-	δ
Mouflon	41	-	δ
Mouflon	42	-	δ
Mouflon	43	-	-
Red deer	44	-	δ
Red deer	45	δ	δ
Mouflon	46	δ	ξ
Mouflon	47	δ	-
Red deer	48	-	δ
Roe deer	49	-	δ
Roe deer	50	-	-
Mouflon	51	-	δ
Red deer	52	-	-
Roe deer	53	δ	ξ
Roe deer	54	-	ξ
Roe deer	55	δ	ξ
Red deer	56	δ	-

Paper VI

E5 nucleotide polymorphisms suggest quasispecies occurrence in BPV-1 sub-clinically infected horses

Abstract

BPV-1 is known as the main causative agent of equine sarcoid, but the virus has also been detected in skin and blood of healthy horses. Previous reports demonstrated the presence of E5 variants in sarcoids of donkeys and horses; we investigated whether this genetic variability might be also found in BPV-1, PBMC associated, of sub-clinically infected horses. With this aim, we analyzed the E5 gene of 21 BPV-1 strains from diseased and sub-clinically infected horses. Our analyses lead us to demonstrate that multiple virus variants can be present in the blood of sub-clinically infected horses, with alternative bases corresponding to either synonymous or non-synonymous codons in the E5 oncogene sequences. The results give support to the proposed existence of "equine adapted" BPV-1 strains with the occurrence of viral variants, resembling quasispecies, in clinically healthy horses with viremia.

Keywords: horse, bovine papillomavirus; oncoprotein; sarcoid; quasispecies

Equine sarcoid is a common skin tumour of considerable importance in veterinary medicine. Bovine Deltapapillomaviruses type 1 and 2 have been recognized as the most important etiologic factor in the development of sarcoids, that are characterized by a non-productive infection resulting in a fibroblastic tumour with minor epithelial component (Nasir and Campo, 2008), in which viral DNA is assumed to exist episomally (Campo, 2002). A wide-spread occurrence of BPV in the horse population was shown (Bogaert et al., 2008) with a 30% BPV infection rate of healthy horses not living in contact with cattle or sarcoid-affected

horses (Bogaert et al 2005). BPV viral DNA has been demonstrated in whole blood of infected cattle (Campo, 1998; de Freitas et al., 2003), and in PBMCs (Peripheral Blood Mononuclear Cells) of sarcoid-bearing horses (Brandt et al., 2008a). In sarcoids, only the early genes are transcribed, in order to maintain viral copy number and to control cell growth (Chambers et al., 2003a). The BPV-1 E5 early protein was one of the first PV oncoproteins to be identified. Despite its limited size and unusual composition, the E5 protein causes cell transformation and contributes to the establishment of viral infection by promoting cell proliferation and immune evasion (Campo, 2002). E5 variants in BPV-1 sarcoid affected horses have been identified (Chambers et al., 2003b) further sequence analyses, performed on BPV-1 E5 gene of viruses isolated from donkey sarcoids, showed the same nucleotide substitutions (Reid et al., 1994). These findings led to the assumption that sequence changes could affect the expression and function of the early proteins and may explain the different pathogenesis of the equine sarcoid compared to papillomas induced by BPV in cattle (Chambers et al., 2003b).

This study aimed at characterizing the E5 gene of BPV-1 of diseased or sub-clinically infected horses, in order to assess the main genetic features of the major oncogene involved in sarcoid pathogenesis. A total of 21 horses, aged between 0 and 26 years and belonging to private owners were sampled. Ten out of twenty one (15/11, 16/11, 17/11, 19/11, 20/11, 21/11, 25/11, 36/11, 37/11, 75f/11) were asymptomatic in regard to sarcoid, but were previously found to be BPV-1 sub-clinically infected in blood; the remaining 11 subjects (2106/11, 1137/10, 231/12, 1407/09, 178/12, 75/11, 688/08, 324/12, 333/12, 322/12, 1219/11) were submitted to surgery for the presence of one or more cutaneous neoplasia.

Blood samples were collected from sub-clinically infected subjects by jugular venipuncture using K3-EDTA-containing tubes and skin samples were collected from sarcoid-bearing horses. PBMCs were separated from whole blood using the Ficoll-Paque® PLUS (GE Healthcare, Uppsala, Sweden) gradient centrifugation following manufacturer‟s instructions.

Genomic DNA was extracted from skin samples and PBMCs using the NucleoSpin® Tissue Kit (Macherey Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer‟s instructions.

Surgical samples were fixed in 10% neutral buffered formalin and routinely processed for histological analysis in order to confirm the nature of the lesion. The microscopic examination of hematoxylin and eosin stained sections confirmed the clinical suspect of sarcoid (Supplemental table 1).

Since BPV-1 E5 variants were already demonstrated in sarcoids of horses and donkeys, we investigated whether this genetic variability can be also found in the blood of healthy horses. PCR amplification of the BPV -1 (499bp) and -2 (497bp) E5 gene was performed as previously described by Brandt and colleagues (2008b). Reactions included negative controls, no template control (NTC) and a sequenced BPV-1 strain used as a positive control. E5 was not detected in negative controls and NTC, while all the sarcoid-bearing subjects were found positive for BPV E5 gene and all the healthy horses confirmed to be sub-clinically infected. The only BPV type identified in the analyzed samples was type 1 giving further support to the evidence that in Europe this accounts for the vast majority of DNA detected (Angelos et al., 1991; Otten et al., 1993). The presence of BPV-1 DNA in the peripheral blood of healthy subjects further proves that blood might be a reservoir of viral DNA as previously reported by Brandt et al., 2008a. As a first screening for the characterization of PCR products, restriction enzyme analyses with MaeIII (Roche Molecular Diagnostics, Mannheim, Germany) have been performed thus being able to detect the nucleotide mutations at position 60 in the E5 gene (Chambers et al. 2003b). Subsequently, the amplicons were purified and directly sequenced by forward and reverse primers. Alignments of the assembled sequences were further viewed and adjusted using BIOEDIT program version 7.2.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Chromatograms were visually inspected showing that those from sarcoid-bearing horses didn’t lead to identify any ambiguous base.

Instead, the analysis of the sequences from 5 healthy subjects (16/11, 17/11, 20/11, 25/11, 37/11) showed clear single-nucleotide polymorphisms (SNPs) at different nucleotide positions in the whole sequence (Figure 1) corresponding either to non- synonymous or synonymous triplets (Table 1). MaeIII digestion emphasized the presence of one or more viral sub-populations in every sample, confirming what was previously evidenced by chromatogram analysis.

The amplification products, suggesting the presence of mixed viral populations, were cloned into the pDriveCloning vector (Qiagen, Hilden, Germany). Positive clones were screened by PCR and MaeIII restriction enzyme digestion. On the basis of the identified pattern, a minimum of 2 and a maximum of 4 recombinant plasmids for each sample were purified and sequenced.

The nucleotide sequences obtained were compared to reference BPV-1 (GenBank accession number: NC_001522) and equine sarcoid (GenBank accession number: JX678969) using CLUSTAL W.

The E5 sequences, amplified from all sarcoid samples, showed the non-coding nucleotide substitution A/G at position 60 (Brandt et al., 2008b; Chambers et al., 2003b). This substitution was the only one identified in samples 324/12 and 1219/11. The single non-coding substitution T/C at position 9 was evident in samples 1407/09, 178/12, 688/08 and 75/11 while stop codon TAA was substituted by TAG in sample 333/12. Samples 333/12, 322/12, 231/12, 1137/10 and 2106/11 showed the coding G/T substitution at position 43, which leads to the change of an alanine with a serine amino acid residue. Four samples (75/11, 688/08, 1407/09, 178/12) were conducible to the prototype sequence of BPV-1 E5 of equine sarcoid (GenBank accession number: JX678969).

Analysis of the complete 135bp E5 gene sequences confirmed that the chromatogram polymorphism was determined by the presence of different viral populations (Supplemental figure 1). A total of 7 different point mutations were identified in the 14 analyzed clones

(Table 1). Besides the mutations at positions 43 G/T and 60 A/G, other five changes at different positions are reported for the first time (Table 1). Our results support the existence of an "equine adapted" BPV strain that infects horses in addition to the prototype BPV. In fact, we found the single non-coding mutation A60G in two out of 5 sub-clinically infected horses, in at least one clone from each healthy sample, while this mutation is constantly identified in sarcoid-bearing horses. This hypothesis is further supported by the findings of Trewby (2012), who stated that the emergence of a slowly evolving virus in horses is unlikely to be a recent phenomenon and cannot be attributed to a single cross-species transmission event.
It has already been documented that the variants detected in equine sarcoids might increase codon usage in equine cells (Chambers et al., 2003b; Nasir et al., 2007; Nasir and Reid, 1999). This is interesting, particularly for the E5 gene, as these variants would be expected to be translated more efficiently in equine cells to increase the cell transformation efficiency (Nasir and Campo, 2008).

According to the Codon Usage Database (http://www.kazusa.or.jp/codon/) it was evident that the synonymous triplets CUG and CUA are used with the same frequency in cattle (43.5‰, 6.1‰ respectively) and horse (44.7‰, 6.0‰ respectively). These data support the hypothesis that „equine-adapted" BPV-1 have the same ability to infect equids of „bovine-infecting" BPV-1. Thus, this finding is in contrast with the assumption that papillomaviruses use rare codons relative to their hosts (Zhao et al., 2005; Zhao et al., 2003).

The genetic heterogeneity of BPV-1 E5 gene found in sub-clinically infected horses might suggest the occurrence of quasispecies (Eigen, 1996). Quasispecies are widely recognized in RNA viruses having error prone mechanisms for replication of nucleic acids. In contrast, DNA viruses use the host replication system with high fidelity, good proof reading capacity, and efficient post replication repair capacity. Previous reports described viral genomic diversity in individual clinical specimens among DNA viruses both in humans and animals (Battilani et al., 2006; Luo et al., 2012) but physiological significance in viral activity have

not yet been fully understood. In humans, the simultaneous presence of multiple viral variants within E6 gene in the same subject, has already been reported for HPV-16 in precancerous cervical biopsies (Vartanian et al., 2008) and in invasive cervical cancer as well as in low-grade squamous intraepithelial lesion (Kukimoto et al., 2013). Our findings may suggest a parallel behavior within the E5 gene of BPV with the presence of quasispecies representing a pre-cancerous state in horses.

In contrast to what it has been detected in sarcoid-bearing donkeys (Reid et al., 1994) and horses (Chambers et al., 2003b), where all samples contained the same E5 variant, in sub-clinically BPV-1 infected horses, we demonstrated the co-existence of both mutated and non-mutated sequences. Our findings might support the hypothesis that these sequence variants possess a particular selective advantage within equids.

In summary, we report for the first time sub-clinically infected horses carrying multiple strains of BPV with a variability detected in the E5 oncogene, which indicates that quasispecies may be common in the context of BPV sub-clinically infected horses. In depth research will be needed to better understand whether sequence variants can be identified as prognostic factors for disease development.

REFERENCES

1. Angelos, J.A., Marti, E., Lazary, S., Carmichael, L.E., 1991. Characterization of BPV-like DNA in equine sarcoids. Archives of Virology 119, 95-109.

2. Battilani, M., Scagliarini, A., Ciulli, S., Morganti, L., Prosperi, S., 2006. High geneticdiversity of the VP2 gene of a canine parvovirus strain detected in a domestic cat. Virology160 352, 22-26.

3. Bogaert, L., Martens, A., De Baere, C., Gasthuys, F., 2005. Detection of bovine papillomavirus DNA on the normal skin and in the habitual surroundings of horses with and without equine sarcoids. Research in Veterinary Science 79, 253-258.

4. Bogaert, L., Martens, A., Van Poucke, M., Ducatelle, R., De Cock, H., Dewulf, J., De Baere, C., Peelman, L., Gasthuys, F., 2008. High prevalence of bovine papillomaviral DNA in the normal skin of equine sarcoid-affected and healthy horses. Veterinary Microbiology 129, 58-68.

5. Brandt, S., Haralambus, R., Schoster, A., Kirnbauer, R., Stanek, C., 2008a. Peripheral blood mononuclear cells represent a reservoir of bovine papillomavirus DNA in sarcoid-affected equines. Journal of General Virology 89, 1390-1395.

6. Brandt, S., Haralambus, R., Shafti-Keramat, S., Steinborn, R., Stanek, C., Kirnbauer, R., 2008b. A subset of equine sarcoids harbours BPV-1 DNA in a complex with L1 major capsid protein. Virology 375, 433-441.

7. Campo, M.S., 1998. Persistent infection by bovine papillomavirus, In: Chen, R.A.a.I.S.Y. (Ed.) Persistent Viral Infections. Wiley Publishing.

8. Campo, M.S. 2002. Animal models of papillomavirus pathogenesis. Virus Research 89,177 249-261.
9. Chambers, G., Ellsmore, V.A., O'Brien, P.M., Reid, S.W., Love, S., Campo, M.S., Nasir, L., 2003a. Association of bovine papillomavirus with the equine sarcoid. Journal of General Virology 84, 1055-1062.
10. Chambers, G., Ellsmore, V.A., O'Brien, P.M., Reid, S.W., Love, S., Campo, M.S., Nasir, L.,2003b. Sequence variants of bovine papillomavirus E5 detected in equine sarcoids. Virus Research 96, 141-145.
11. de Freitas, A.C., de Carvalho, C., Brunner, O., Birgel- Junior, E.H. 2003. Viral DNA sequences in peripheral blood and vertical transmission of the virus: a discussion about BPV-1. Brazilian Journal of Microbiology, 76-78.
12. Eigen , M. 1996. On the nature of quasispecies. Trends in Mcrobiology 4, 216-218.
13. Knottenbelt, D.C., 2005. A Suggested Clinical Classification for the Equine Sarcoid. Clinical Techniques in Equine Practice 4, 278-295.
14. Kukimoto, I., Maehama, T., Sekizuka, T., Ogasawara, Y., Kondo, K., Kusumoto-Matsuo, R., Mori, S., Ishii, Y., Takeuchi, T., Yamaji, T., Takeuchi, F., Hanada, K., Kuroda, M., 2013. Genetic variation of human papillomavirus type 16 in individual clinical specimens revealed by deep sequencing. PLoS One 8, e80583.
15. Luo, C., Hirsch, H.H., Kant, J., Randhawa, P., 2012. VP-1 quasispecies in human infection with polyomavirus BK. Journal of Medical Virology 84, 152-161.
16. Martens, A., De Moor, A., Demeulemeester, J., Ducatelle, R., 2000. Histopathological
197 characteristics of five clinical types of equine sarcoid. Research in Veterinary Science 69,
198 295-300.
17. Nasir, L., Campo, M.S., 2008. Bovine papillomaviruses: their role in the aetiology of cutaneous tumours of bovids and equids. Veterinary Dermatology 19, 243-254.
18. Nasir, L., Gault, E., Morgan, I.M., Chambers, G., Ellsmore, V., Campo, M.S., 2007. Identification and functional analysis of sequence variants in the long control region and the E2 open reading frame of bovine papillomavirus type 1 isolated from equine sarcoids. Virology 364, 355-361.
19. Nasir, L., Reid, S.W., 1999. Bovine papillomaviral gene expression in equine sarcoid tumours. Virus
esearch 61, 171-175.
20. Otten, N., von Tscharner, C., Lazary, S., Antczak, D.F., Gerber, H., 1993. DNA of bovine papillomavirus type 1 and 2 in equine sarcoids: PCR detection and direct sequencing. Archives of Virology 132, 121-131.
21. Reid, S.W., Smith, K.T., Jarrett, W.F., 1994. Detection, cloning and characterisation of papillomaviral DNA present in sarcoid tumours of Equus asinus. Veterinary Record 135, 430-432.
22. Trewby, H., 2012. Three studies showing the importance of quantitative methods in investigation of veterinary infectious disease. University of Glasgow,
23. Vartanian, J.P., Guetard, D., Henry, M., Wain-Hobson, S., 2008. Evidence for editing of human papillomavirus DNA by APOBEC3 in benign and precancerous lesions. Science 320, 230-233.
24. Zhao, K.N., Gu, W., Fang, N.X., Saunders, N.A., Frazer, I.H., 2005. Gene codon composition determines differentiation-dependent expression of a viral capsid gene in keratinocytes in vitro and in vivo. Molecular Cell Biology 25, 8643-8655.
25. Zhao, K.N., Liu, W.J., Frazer, I.H., 2003. Codon usage bias and A+T content variation in human papillomavirus genomes. Virus Research 98, 95-104

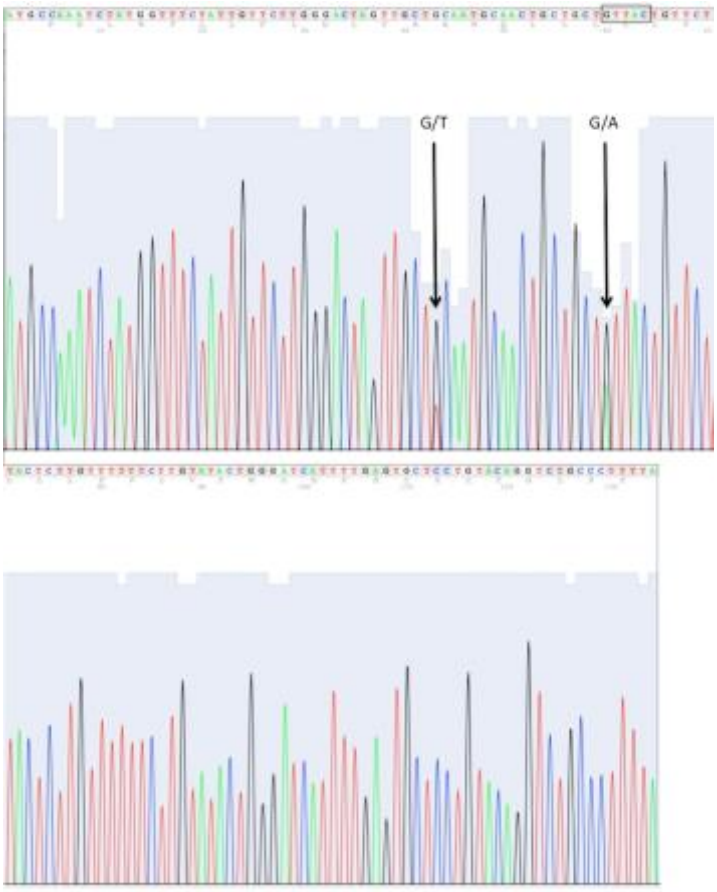
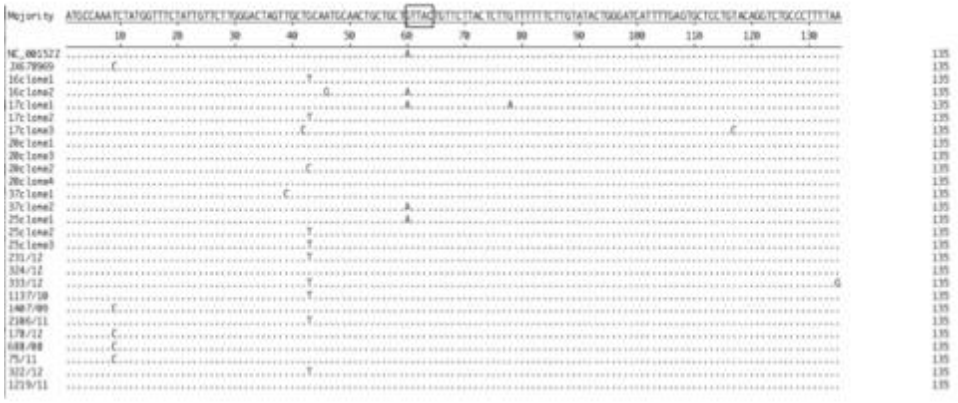


Figure 1- Unprocessed raw chromatogram depicting minor peaks, representative of the quasispecies' genotypes, that can be seen under the highlighted major peaks from a BPV-1 sub- clinically infected horse. MaeIII restriction site (↓GTNAC□ is evidenced (boxed) at position 60 of the E5 sequence where a mixed population is identified by the double peaks.

Table 1- Nucleotide substitutions in clones of BPV-E5 in sub-clinically infected horses. Different nucleotides are indicated by a letter; the nucleotides identical to the reference sequence (GenBank accession number NC_001522) are indicated as dashes while the nucleotide differing are written. Nucleotide changes which result in amino acid substitutions are indicated with the deduced amino acid changes. Nucleotide position is referred to the E5 gene sequence.

Nt. E5 gene	39	42	43	46	60	78	117
NC_001522	T	T	G	A	A	G	T
16 clone 1	-	-	T	-	G	-	-
16 clone 2	-	-	-	G	-	-	-
17 clone 1	-	-	-	-	-	A	-
17 clone 2	-	-	T	-	G	-	-
17 clone 3	-	C	-	-	G	-	C
20 clone 1	-	-	-	-	G	-	-
20 clone 2	-	-	C	-	G	-	-
20 clone 3	-	-	-	-	G	-	-
20 clone 4	-	-	-	-	G	-	-
37 clone 1	C	-	-	-	G	-	-
37 clone 2	-	-	-	-	-	-	-
25 clone 1	-	-	-	-	-	-	-
25 clone 2	-	-	T	-	G	-	-
25 clone 3	-	-	T	-	G	-	-
aminoacid substitutions	Val→Val	Ala→Ala	G/C Ala→Pro G/T Ala→Ser	Met→Val	Leu→Leu	Leu→Leu	Cys→Cys



Supplemental figure 2- Complete alignment of BPV-1 E5 gene sequences of reference strains, clones obtained from sub-clinically infected animals and sarcoid bearing horses. MaeIII restriction site (↓GTNAC is identified only in sarcoid bearing horses and in several BPV-1 variants (clones) of the mixed populations found in sub-clinically infected animals.

Supplemental table 1- Sarcoid samples and their localization. Histological examination confirmed the cutaneous tumors as sarcoids in all 11 cases. Based on Knottenbelt (2005), the clinical pattern was fibroblastic in 5 cases, verrucous in 2, mixed in 2, 1 nodular and 1 occult. Histologically, according to Martens et al. (2000), hyperkeratosis and epidermal hyperplasia varied from mild (3 cases) to moderate/marked (6 cases); epidermis showed thinning in 2 cases and partial surface ulceration in 5; cell palisading at the dermal-epidermal junction was evident in 8 cases and rete pegs in 6; as for the dermal proliferation, neoplastic fibroblast were densely packed in 9 cases, with typical whirling pattern in 7 cases.

Sample n°	Type of lesion	Localization
75/11	Mixed	inguinal area
1137/10	Verrucous	neck
1219/11	Mixed	ventral abdomen
2106/11	Verrucous	pastern
178/12	Fibroblastic	chest
231/12	Fibroblastic	inguinal area
688/08	Fibroblastic	chest
1407/09	Fibroblastic	inguinal area
324/12	Occult	chest
333/12	Nodular	perinaeum
322/12	Fibroblastic	ventral abdomen

IV
GENERAL DISCUSSION AND CONLCUSIONS

Animal PVs have provided powerful models for antiviral vaccines development for humans (Campo, 1997), this is particularly true for some animal PVs including COPV (Teifke et al., 1998), CRPV (Rous et al., 1935), Rhesus papillomavirus (RhPV) (Ostrow et al., 1990), and BPV (Campo et al., 1992) that share with the High Risk HPVs the ability to transform their host cells and lead to the development of cancer. During the past 50 years, studies of BPV-associated lesions have been useful in helping to understand the complex relationship between HPVs and human cancer. In addition, unlike HPVs, BPVs were found to readily transform cells *in vitro*, and BPV-transformed cells have been valuable in determining how PVs cause cancer (Meischke, 1979).

The identification of the PV viral types, causing infections in animals, is also necessary to understand the geographical distribution and pathology of PV and to develop proper prophylactic and therapeutic measures. Despite a broad documentation of the incidence of BPVs in cattle herds in various regions of the world (Singh et al., 2009; Schmitt et al., 2010; Freitas et al., 2011; Carvalho et al., 2012) scant information about the types distribution in Italy is available (Borzacchiello et al., 2003a, 2003b; Roperto et al., 2014; Grindatto et al., 2015). The survey described in PAPER I contributed to fill the gap regarding the lack of information on the BPVs circulating in Emilia Romagna region and hypothetically on the Italian territory. This first study depicts a situation of heterogeneous infections, where the frequency of the combinations observed reproduces and may depend on the diffusion of the single genera in the country. We identified all four BPVs genera, hence types 1, 2, 3, 6, 7, 8, 9, 10 and 12 in a total of xx samples. BPVs 6, 7, 8, 10 and 12 where described for the first time in Italy.

The multiplicity of viral types identified, even inside single herds, add significant knowledge about the prevalence and diversity of BPV infection. This information may be used in future

studies aiming at the development of more specific treatment and diagnostic methods. Our results confirm what was previously observed in other countries about the high frequency of co-infections (Schmitt et al., 2010; Carvalho et al., 2012; Santos et al., 2014), in fact more than a half of the samples harbored different genera, hence PV types. The high prevalence of co-infections observed in our and in other studies, may suggest that different BPV types do not interfere with each other even though it is still unknown whether the lesions are caused by one type only and the other types are passengers, whether they are biologically active and what their viral load is (Schmitt et al., 2010). In general, the identification of multiple BPV infections may contribute to the understanding of the epidemiological, and the clinical features as well as the oncogenic potential of the virus and the relationship between virus and co-factors in developing cutaneous papillomatosis in cattle.

A more detailed characterization of the genomes of BPV-7 variants amplified from nine teat skin lesions of dairy cows collected during the previous survey is performed in PAPER II. Direct sequencing of the entire genome was feasible only for two viral strains, the Italian variants BPV-7 #215 and #221 that showed major sequence differences in the long control region (LCR) and in the L2 gene compared to the Japanese reference strain, where it was firstly identified in diseased and healthy teat skin samples (Ogawa et al., 2007). In order to better define the stability of the variants, particular attention was given to the L2 and LCR amplicons of the 7 remaining partially sequenced samples. The deletions and insertions identified in our specimens are consistent with previous findings in different papillomavirus types identified in *Arctiodactyla* (Scagliarini, et al., 2013; Zhu et al., 2014; Dong, et al., 2013), but genomic duplication, insertion and deletion have been also detected in the LCR region of HPV- 6 and -11 where these rearrangements have been shown to be associated with clinically malignant or malignant tumors (Kasher et al., 1988; Chin et al., 1989; Favre et al., 1990; Rübben et al., 1992; Kitasato et al., 1994;).

Our findings evidenced intra-type variability of BPV-7 L2 and further studies on variants will clarify the consequences of the amino acid deletions on the antigenicity and will add information on functional motifs of this PV structural protein. Analyses of the modified LCR region by functional assays suggested that activity of this regulatory region was in some cases enhanced (Wu and Mounts, 1988) while in others, insertions and duplications did not influence the protein expression (Combrinck et al., 2012).

Our identification contributes to the understanding of BPV and PV replication and transcription, confirming that deletions but also insertions can be observed in other BPV-positive papilloma samples. Whether the genetic BPV-7 variants possess distinct biological properties *in vivo*, remains to be determined and further studies will be necessary to detect the functionality of the deleted genome to confirm if this kind of genome's deletions have implications in disease progression.

The objective of Paper III was to determine the causes of several outbreaks of malignant proliferative skin diseases in domestic ruminants in Sicily during 2011-2014 that were clinically diagnosed as papillomatosis. This study enlighted he presence of different BPV genera and types (i.e BPV1, 2, 6, 7, 8) both as single or multiple infections, confirming what previously had been reported in Paper I for Emilia Romagna. Due to the peculiar features of the tumor-like lesions found in Sicily pathological samples were also screened with a mini array test for the rapid detection of zoonotic poxviruses developed within POC4PETS FP7 project. Analysis demonstrated papillomavirus co-infections with parapoxviruses in seven out of twelve cows. Furthermore, two cattle that showed the presence of papillomavirus particles by EM were also co-infected with zoonotic CPXV belonging to *Orthopoxvirus* genus. Even if it was not possible to conclude that CPXV was the causal agent of the malignant lesions for its low genomic load showed by Real time PCR performed on samples. On the other hand the presence of CPXV DNA identified in the pathological samples of diseased cattle and goats from the same area lead us to speculate that the virus may use these animal species as

reservoirs to spread and persist in the environment through the scab material produced by epitheliotropic BPVs containing high titers of stable viruses that can remain infectious long enough to sustain indirect transmission of both viruses. Papillomavirus and poxvirus infections recognize the same risk factors caused by highly resistant agents, in addition both viruses have a particular tropism for epithelial cells, but they do not necessarily require direct contact between infected and susceptible host to be transmitted. Poxvirus zoonoses fall into the category of neglected ones and their incidence in animals and humans is often unknown or greatly underestimated, therefore early recognition of the etiologic agents allows proper control measures and disease management strategies to prevent disease spread among animals and humans in the case of zoonotic viruses.

The results of PAPER III suggest a further biological role of BPVs which can contribute to the spread of other epitheliotropic viruses, including those responsible of zoonoses, by promoting their target cells proliferation in a Trojan horse fashion.

Skin tumors are also common in wildlife species, in particular the genomes of several cervid papillomaviruses have been sequenced, such as the Deer (White-tailed deer – *Odocoileus virginianus*) Papillomavirus, the Reindeer (*Rangifer tarandus*) Papillomavirus, the European Elk (*Alces alces*) Papillomavirus and the Western roe deer (*Capreolus capreolus*) papillomavirus. The majority of cervid papillomaviruses belong to the *Delta* genus, whose components have the common feature of inducing the development of skin tumors with a marked connective tissue component. Investigation on a firm cutaneous tumor of a male red deer shot in the Italian Alps in Brescia province is described in PAPER IV. Viral particles, identified by morphologic features as PV were observed by negative stain electron microscopy, histological examination classified the tumor as a fibropapilloma and a subsequent molecular investigation demonstrated the presence of both BPV1 and CePV1 DNA. The coinfection of a red deer with BPV1 is not surprising and confirms the host-jumping ability of the virus. . The genetic organization of CePV1exhibits a typical *Delta*

papillomavirus architecture, in addition CePV1 is the closest relative of CcaPV1 identified in roe deer (*Capreolus capreolus*), where red deer and roe deer belong to the 2 different subfamilies *Cervinae* and the *Capreolinae*. Our data therefore indicate that these PVs belong to the same ancestral lineage and infection of a different host can be the result of a host switch event. In fact, even though co-divergence plays an important evolutionary role in PVs, it is not the only event that shaped PV diversification (Gottschling et al., 2011), since other events such as duplication and host switching can disrupt the topological congruence between the phylogenetic trees of *Delta* papillomaviruses and their hosts. Interspecies transmission of viruses is a broadly accepted phenomenon but it is still debated whether it requires adaptation to a new host during the early stages of infection or whether transmission itself could be a largely random process (Dennehy et al., 2006). PAPER V regards a molecular investigation of cutaneous and mucosal healthy samples collected from sites naturally exposed to microlesions demonstrated the presence of a CePV1 and BPV1 co-infection in a red deer. In the same study, amplification of *Delta* (BPV1, 2, EqSarc BPV1) and *Xi* BPVs widen information on biology of the virus providing new data about host range, tropism and dissemination. *Xi* genus is comprised of pure epitheliotropic viruses affecting cattle; to our knowledge this identification as a single infection or in association with *Delta* papillomavirus types 1 and 2 serves as the first description of their ability to infect other hosts, in particular asymptomatic ones. Among Deltapapillomavirus types, the sequenced E5 gene revealed the presence of BPV1, 2 and BPV1 EqSarc1 E5 gene isolates (GenBank accession JX678969), which presents a single nucleotide exchnge that was suggested to increase codon usage in equine cells (Chambers et al., 2003). This mutation has been detected in sarcoid-bearing horses (*Equus caballus*) but not in cattle; furthermore, we identified the Eqsarc1 variant in non-equid species. This finding might suggest an "equine adaptation" of the virus variant (Yuan et al., 2007; Nasir and Campo 2008), as this is known to cause lesions in horses, but it was also demonstrated to infect other species subclinically. The ability of Eqsarc1 variant to

asymptomatically infect other species suggests that wildlife could be a reservoir for this particular PV, and equine sarcoids could be the result of repeated cross-species infection (Trewby et al., 2014) by an ancient bovine PV subsequently adapted to both *Bovidae* and *Cervidae*. A similar situation was described in cats, where the Deltapapillomavirus FeSarPV causes sarcoids but is also asymptomatic in bovines; therefore, cross-species infection has been suggested (Munday and Knight 2010).

More evidence to the presence of an "equine adapted" BPV1 strain that infects horses in addition to the prototype BPV is given in Paper VI, where the genetic heterogeneity of BPV1 E5 gene found in sub-clinically infected horses might suggest the occurrence of quasispecies (Eigen, 1996). This hypothesis is further supported by the findings of Trewby (2012), who stated that the emergence of a slowly evolving virus in horses is unlikely to be a recent phenomenon and cannot be attributed to a single cross-species transmission event. In contrast to what it has been detected in sarcoid-bearing donkeys (Reid et al., 1994) and horses (Chambers et al., 2003b), where all samples contained the same E5 variant, in sub-clinically BPV1 infected horses, we demonstrated the co-existence of both mutated and non-mutated sequences. Our findings might support the hypothesis that these sequence variants possess a particular selective advantage within equids.

Current classification system assumes that the host species, in which a PV was firstly detected is in fact the original host, and the identified types are therefore named after it. Both Paper IV and V, in accordance with Bravo et al. (2010), claim that the current PV classification system should be revised and an alternative naming approach should be considered, since host specificity is not always strictly maintained in animal papillomaviruses.

In summary, we bring to light a kaleidoscopic situation of BPVs infection in different domestic and wild species. Further studies will be needed to identify other species that can be sub clinically infected, as well as to better understand their role in infection maintaining and transmission. The latter aspect is also fundamental in clinically infected subjects, in fact,

although a causal relationship between BPV1 and equine sarcoids is well established, how *Equidae* become infected with BPV1 remains unknown. In general, understanding the phylogenetic relationships and mechanisms driving PVs diversification will provide better insight into viral evolution and virus–host interactions.